Isothiocyanate metabolism in generalist lepidopteran herbivores

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Katharina Schramm

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Gutachter: Prof. Dr. Jonathan Gershenzon; Max Planck Institut für Chemische Ökologie, Jena
PD Dr. Klaus Appenroth; Universität Jena, Jena
Prof. Raymond Barbehenn, PhD; University of Michigan, Ann Arbor, USA

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Research is the process of going up alleys to see if they are blind.

Marston Bates

1 General Introduction

The relationships between plants and insects include a number of interactions of a very different nature. Plants can serve as host, food, nursery, shelter, lure, trap, and/or messenger for insects. In return, insects may be friends or enemies, herbivores, defenders, pollinators, seed dispersers or food as well. Depending on the insect species, plants are more or less well defended against all sorts of attack with a whole arsenal of defensive properties. Physical defences include thorns, surface waxes, tough leaves, resins, latexes and glands filled with sticky or irritating secretions (Fernandes, 1994; Rosenthal & Kotanen, 1994; Boege & Marquis, 2005; Hanley et al., 2007). Additionally, plants may produce a large variety of secondary metabolites which to insects may be repellent or lethal (Fraenkel, 1959; Howe & Westley, 1988; Schoonhoven et al., 2005). Generally, there is a distinction between constitutive and inducible defence compounds. Constitutive defences are continuously present in plant tissues, usually at fairly high concentrations. Typical representatives are polyphenols, tannins, saponins, lignins and terpene-containing essential oils. Inducible defences usually are produced only in case of an attack on the plant by herbivores or pathogens. Sometimes these metabolites are present prior to attack on the plant, but their concentration is rapidly increased when an attack ensues. It is widely believed that inducibility is an evolutionary solution to the cost of defence production. Since many induced defences contain nitrogen or sulphur, and nutrients are sometimes in short supply, or require large amounts of fixed carbon and energy in their manufacture or storage, they are only produced when needed.

An interesting intermediate between constitutive and induced plant defences are activated defence systems or phytoanticipins. Here, the actual defence compound is generated only when plant tissue is injured. Such systems usually are made up of an inactive precursor compound and an activator (often an enzyme) which are physically separated in intact plant tissue. In case of tissue damage, the components come into contact and the precursors are transformed to biologically active metabolites by the activators. The advantages of such systems are that defence compounds which may potentially be unstable or phytotoxic are only produced in case of attack. Examples of such systems are the cyanogenic glycosides, the alliines (found exclusively in plants of the genus Allium) and the glucosinolates (Fig. 1), present in plants of the Brassicales and of the genus Drypetes (Euphorbiaceae) (Block, 1992; Rodman et al., 1996; Gleadow & Woodrow, 2002).

Herbivorous insects have evolved numerous strategies to overcome plant defences. It is reasonable to distinguish between specialist and generalist insect herbivores although the definitions are not easy to enforce as there are many intermediate feeding strategies. Generalists usually feed from a broad range of host plants, offering an equally broad variety of defensive compounds to deal with. Specialists, on the other hand, typically feed on a limited number of plant species which often are characterised by sharing a specific class of defence compounds. While specialists commonly have evolved specialised adaptations, either in behaviour or physiology, to deal with their host's defences, generalist feeders are faced with a diversity of defences, both physical and chemical which need to be met. Thus, generalists usually have a multitude of general behavioural patterns (such as browsing, palatability testing or discontinuous feeding) as well as an impressive spectrum of generalist detoxification enzymes (such as cytochrome P450s, mixed functional oxidases or glutathione-*S*-transferases) available. Generalists are much more flexible when it comes to choosing host plants, but run a higher risk of toxication. In contrast, specialists, whilst well prepared for their hosts' defences, may be more severely affected by a lack of host plants. Amongst lepidopterans, the generalist feeding strategy is rarer than the specialised model. *Spodoptera littoralis* Boisduval (Noctuidae), the Egyptian cotton bollworm, study model organism used throughout most of this thesis, is a truly polyphagous generalist species. Host plants span more than 40 different species and include many important crop plants such as cotton, different species of Leguminosae and members of the crucifer plant family (Brassicales) (Ellis, 2004; Venette et al, 2003).

The glucosinolate myrosinase system

Plants of the order Brassicales possess an activated defence system consisting of at least two components: glucosinolates, the non-toxic precursor compounds and myrosinases, their activating enzymes. In intact plant tissue, the two components are spatially separated, but when the tissue gets damaged, e.g. by a chewing herbivore, they come together. The myrosinases hydrolyse the glucosinolates forming an array of biologically active compounds (Fig. 1) (Matile, 1980; Rask *et al.*, 2000; Halkier & Gershenzon, 2006). Glucosinolates are structurally diverse, with over 120 different compounds identified so far (Fahey *et al.*, 2001; D'Auria & Gershenzon, 2005; Grubb & Abel, 2006). The basic sceleton of glucosinolates consists of a backbone of glucose attached to a sulfonated aldoxime with a variable side chain (examples in Fig. 2). The structure of the side chain is mostly based on the amino acid from which it is derived (aromatic: tyrosine, phenylalanine; aliphatic: methionine, leucine, isoleucine, valine or alanine; indolic: tryptophan), coupled with a number of possible chain elongation and modification steps.

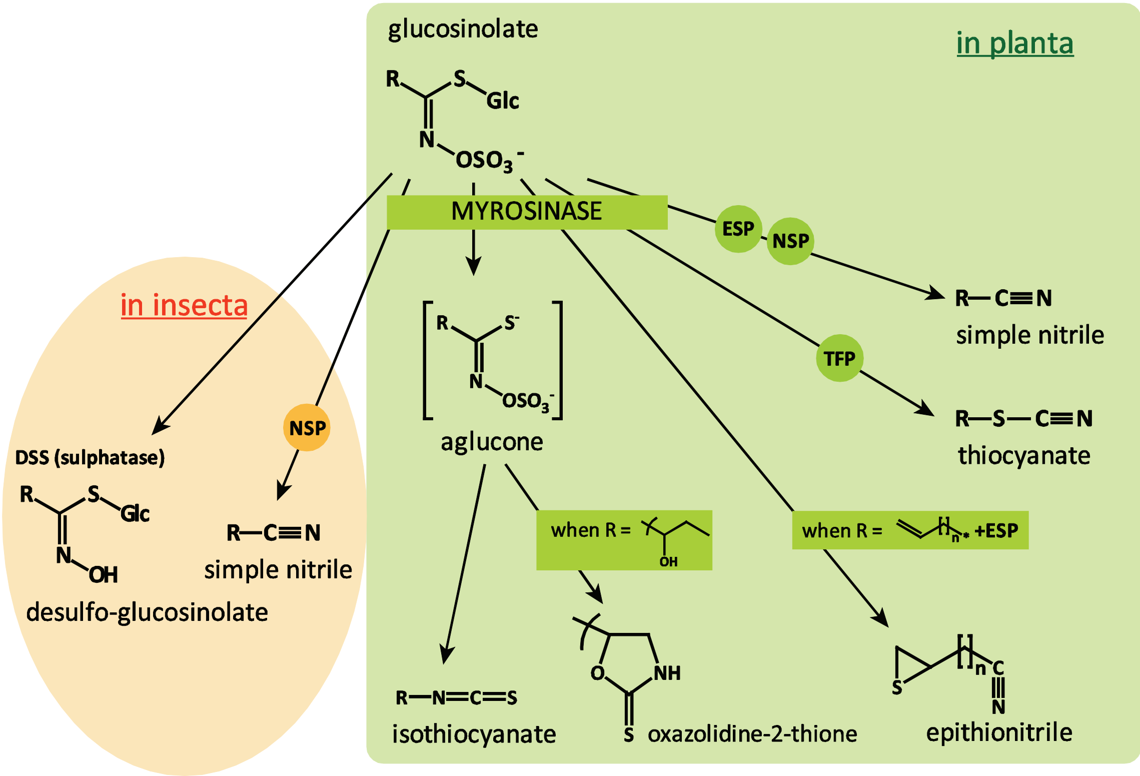

Fig. 1: Glucosinolate hydrolysis in insects and plants.

When glucosinolates and myrosinases get into contact, the myrosinases hydrolyse the glucosinolates, except when an insect sulphatase (DSS) like the one from *Plutella xylostella* is involved. There the glucosinolates get desulphated so that they are no longer myrosinase substrates. In planta the outcome of hydrolysis depends on the presence of specifier proteins like ESP and NSP and/ or the structure of the glucosinolate side chain (as for epithionitriles). Pieris rapae NSP leads to formation of simple nitriles instead of isothiocyanates in the presence of myrosinases.

When glucosinolates are hydrolysed, the glucose is removed and the aglucone is typically turned into an isothiocyanate by a Lossen rearrangement. However, depending on the structure of the side chain and conditions in the plant, such as pH or the presence of specifier proteins or cations, other hydrolysis products may occur: simple nitriles, epithionitriles, thiocyanates and oxazolidine-2-thiones (Fig. 1; (Wittstock & Burow, 2010). Thus, a variety of hydrolysis products can be produced from a single parent glucosinolate, which increases the diversity of hydrolysis products way beyond that of the glucosinolates. The biological significance of this structural diversity is not yet fully understood, but it likely contributes to the toxicity of the glucosinolate myrosinase system as a plant defence. Possibly, the combination of structures differing in toxicity increases its activity against a greater range of enemies.

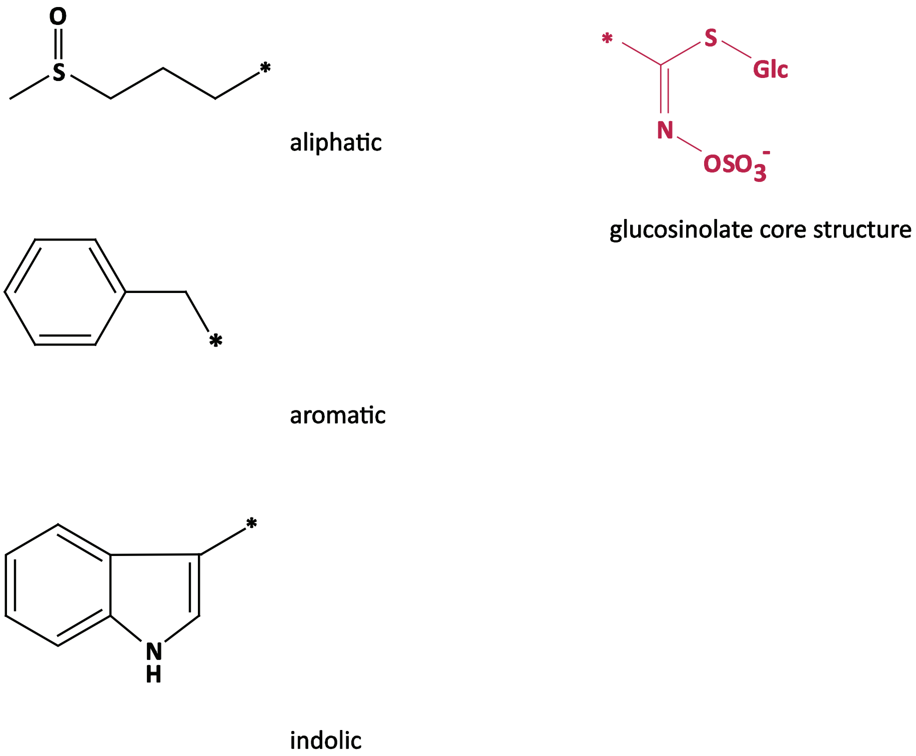

Fig. 2: Glucosinolates have variable side chains.

Depending on the amino acid they are derived from, glucosinolates can be classified as aliphatic, aromatic or indolic. Each class has a considerable number of members, owed to chain elongation steps and other possible modifications during biosynthesis. In total, over 120 different glucosinolates have been described.

The most frequently studied of the glucosinolate hydrolysis products, the isothiocyanates, are toxic to a wide variety of organisms, including insects, pathogens and bacteria. Toxicity towards insects is comparable to that of synthetic insecticides (Wittstock et al., 2003). The activity of isothiocyanates is conferred by the isothiocyanate function itself, which has been shown to cleave disulfide bonds in proteins and react with amino acids in vitro (Kawakishi & Kaneko, 1987). However, the rest of the molecule influences its polarity and volatility, and thus the ability of the isothiocyanate to access various targets in an organism. Increasing molecular weight elevates toxicity (Borek et al., 1998) and aromatic isothiocyanates are more toxic than aliphatic ones (Wadleigh & Yu, 1988). Since most glucosinolate containing plants produce a whole spectrum of different glucosinolates, herbivores will usually be confronted with a range of different isothiocyanates which may exhibit increased toxicity compared to a single isothiocyanate (Wadleigh & Yu, 1988; El Sayed et al., 1996; Borek et al., 1998).

The effects of isothiocyanates on insect herbivores range from long term effects, such as increased mortality (Wadleigh & Yu, 1988), extended development times (Agrawal & Kurashige, 2003; Burow et al., 2006), reduced growth, reduced weight gain and prolonged pupation (Burow et al., 2006; Ulmer & Dosdall, 2006) to immediate effects such as repellence and feeding deterrence (Burow et al., 2006; Ulmer & Dosdall, 2006). Where investigated, it was shown that isothiocyanate toxicity is correlated to molecular structure.

Isothiocyanates and their metabolism in mammals and insects

The glucosinolate-myrosinase system has been studied extensively because of its impact on human nutrition. Glucosinolates and their hydrolysis products exert considerable influence on the palatability and taste of many brassicaceous plants, including broccoli, cauliflower, cabbage and radishes. On top of that, isothiocyanates are believed to possess anti-carcinogenic properties, which is why the intracellular effects of isothiocyanates and isothiocyanate metabolism have been studied in detail. In humans, isothiocyanates are metabolised via the mercapturic acid pathway and excreted predominantly as *N*-acetylcysteine (NAC) conjugates in the urine and bile (Habig *et al.*, 1974; Conaway *et al.*, 2002). A proposed model of isothiocyanate metabolism is displayed in Fig. 3 (Traka & Mithen, 2009).

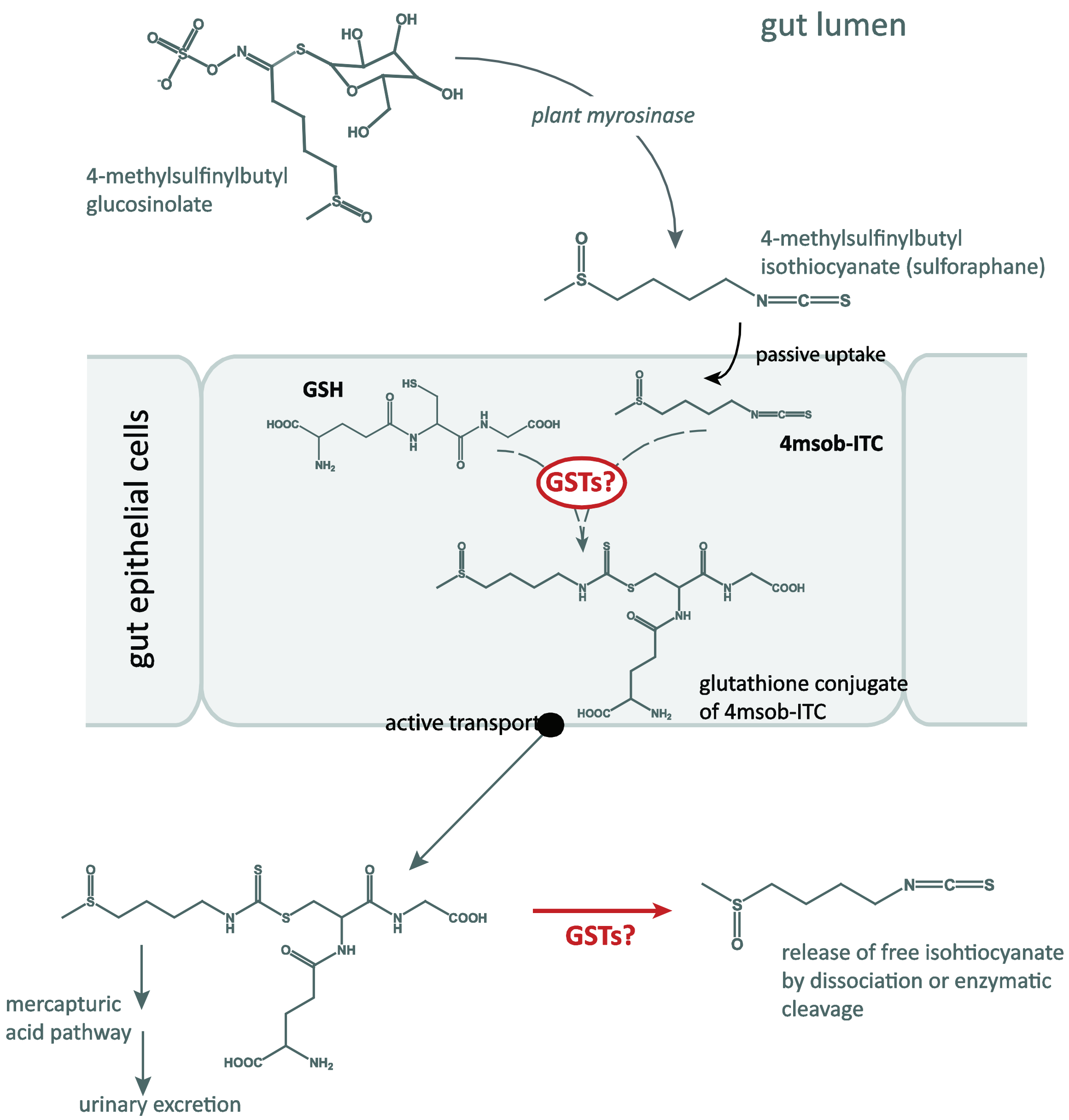

Fig. 3: Proposed route of isothiocyanate metabolism in humans (after Traka & Mithen, 2009).

Intact glucosinolates are ingested and hydrolysed to isothiocyanates by gut microbial myrosinases, or plant myrosinases that act during ingestion. Isothiocyanates are passively taken up by gut epithelial cells where they are conjugated with glutathione immediately. This conjugation may occur spontaneously or with catalytic help from glutathione-S-transferases (red circle). Glutathione isothiocyanate conjugates are actively excreted from the cells and subsequently metabolized via the mercapturic acid pathway and renally excreted. Or, isothiocyanates are released from the conjugate. As free isothiocyanates, they are active as anti-carcinogenics.

Since brassicaceous plants are often processed for consumption in ways that deactivate the plant myrosinases (e.g. boiling), the release of isothiocyanates in humans is often believed to be catalysed by hydrolytic enzymes of the endosymbiotic bacteria in the intestine. Isothiocyanates are then passively absorbed into the gut epithelial cells, where they are conjugated with glutathione. This reaction can occur spontaneously or it can be catalysed by a class of enzymes called glutathione-*S*-transferases (GSTs). Glutathione-isothiocyanate conjugates are subsequently exported from the cells and moved around the body in the blood circulation system. Finally, they are deposited in the kidneys, where they are converted to NAC conjugates before being excreted (Traka & Mithen, 2009). In other mammals, isothiocyanates are metabolised similarly, yet the final product is not necessarily an NAC conjugate. In guinea pigs and rabbits for example, benzyl-ITC is excreted as the mercaptopyruvic acid conjugate, whereas dogs excrete the glycine conjugate (hippuric acid). Rats, like humans, excrete the NAC conjugate (Conaway *et al.*, 2002).

In insect herbivores, glucosinolates are metabolised in a variety of different ways. Insect feeders which specialise on brassicaceous plants, such as the caterpillars of the cabbage white butterfly *Pieris rapae* or those of the diamondback moth *Plutella xylostella*, have developed special adaptations that circumvent the production of isothiocyanates. *P. rapae* produces an enzyme which redirects glucosinolate hydrolysis away from isothiocyanates and instead promotes formation of simple nitriles (Wittstock *et al.*, 2003). *P. xylostella* possesses a sulphatase which cleaves the sulphate off the glucosinolate. The myrosinases do not accept these desulfo-glucosinolates as substrates and unhydrolysed desulfo-glucosinolates are excreted with the faeces (Ratzka *et al.*, 2002). The avoidance of isothiocyanate formation does not mean that those species are insensitive to these toxic plant metabolites. Both *P. xylostella* and *P. rapae* larvae experience increased mortality and reduced growth when forced to feed on isothiocyanates (Li *et al.*, 2000; Agrawal & Kurashige, 2003). Another strategy to avoid toxic isothiocyanates is the sequestration of intact glucosinolates as was found in the Turnip sawfly *Athalia rosae*, the Harlequin bug *Murgantia histrionica* and the Cabbage aphid *Brevicoryne brassicae*.

However, these strategies are found in specialised insect species. Generalist insects which feed on plant species with and without glucosinolates might not be expected to have such sophisticated means of glucosinolate processing. However, much fewer studies on generalists have been carried out. An exception is the locust, *Schistocerca gregaria,* which possesses an inducible sulphatase that works similarly to the one found in *P. xylostella*, but is only activated when locusts feed on glucosinolate containing plants (Falk & Gershenzon, 2007). It is known that isothiocyanates induce GST activity in generalist insect herbivores such as aphids (Francis *et al.*, 2005) and butterfly larvae (Yu, 1982; Yu, 1983), with isothiocyanate structure and concentration playing a significant role in this process (Yu, 1983; Wadleigh & Yu, 1988).

Yet, whether conjugation with GSH is a significant route of metabolism in insects and whether this process is catalysed by GSTs remains to be elucidated. Wadleigh & Yu (1988) showed that free GSH disappears when used as a substrate in assays with GSTs from *S. frugiperda* and isothiocyanates. They thus reasoned that those GSTs catalyse the conjugation of isothiocyanates with GSH. It has not yet been investigated, whether conjugation with GSH is a major route of isothiocyanate detoxification in generalist insect herbivores, whose products are excreted in the faeces, and whose enzymes play a role in this process.

The role of GSTs and glutathione in isothiocyanate metabolism in mammals

While the participation of GSH in isothiocyanate metabolism in humans and other mammals cannot be doubted, it is still not completely clear whether GSTs play a role in this process. GSTs from a variety of organisms are capable of conjugating GSH to isothiocyanates of different chemical structure (Kolm *et al.*, 1995; Meyer *et al.*, 1995; Nutricati *et al.*, 2006; Wiktelius & Stenberg, 2007). Yet, all studies investigating the enzymatic metabolism of isothiocyanates share a similar shortcoming: enzyme assays are usually conducted at conditions giving the least non-enzymatic background. In general, this is a perfectly sensible approach in performing enzyme assays. However, since isothiocyanates and GSH rapidly react with each other without any enzymatic assistance (Ketterer, 1982), it is uncertain whether enzymatic activity is needed to achieve conjugation of isothiocyanates with GSH *in vivo*. In fact, if the conditions present in cells (physiological pH, high concentrations of GSH) are approximated in *in vitro* assays, non-enzymatic background is too high to show enzymatic activity (Meyer *et al.*, 1995). Thus, it remains an open question whether GSTs are involved in isothiocyanate conversion to mercapturic acid in mammals.

Maybe one hint towards a relatively minor contribution of GSTs in this process is the importance of GSH as anti-oxidant. Isothiocyanates are known to cause oxidative stress, especially at higher concentrations (Zhang *et al.*, 2005). Yet, after isothiocyanates have passively entered human or rat cells, a range of reactions occurs that oppose oxidative stress, many of which are dependent on, or in other ways related to GSH. For example, isothiocyanates immediately are conjugated with GSH. In this way, isothiocyanates are rapidly accumulated intracellularly, mostly in the form of GSH conjugates. The conjugates stimulate Antioxidant Response Elements (ARE) which activate a number of different detoxification and antioxidant-related genes. These include genes of different detoxification enzymes like GSTs and quinone reductase and that of γ-glutamylcysteine synthase, the rate-limiting enzyme of GSH biosynthesis (Zhang & Talalay, 1994; Ye & Zhang, 2001). The induction of this last enzyme eventually leads to a rise in intracellular GSH beyond the normal level.

This is important for two reasons: it repletes GSH levels after export of conjugates from the cell has caused extensive and rapid depletion of glutathione (Zhang, 2000; Zhang *et al.*, 2005). Secondly, it enables the uptake and thus metabolism of more isothiocyanate. Isothiocyanate uptake is positively correlated with time and isothiocyanate concentration (Zhang, 2000; Zhang, 2001; Zhang & Callaway, 2002) and apparently enhanced in the presence of GSTs, supporting a contribution of GSTs to isothiocyanate conjugation. Yet, over extended periods of exposure (24 h), the effect brought on by GSTs disappears (Zhang, 2001), once more putting the significance of these enzymes for the process of isothiocyanate metabolism to question.

It appears that glutathione plays an important role in the intracellular metabolism of isothiocyanates. It is generally believed that GSTs are necessary for mediating GSH isothiocyanate conjugation, but this may simply be because it would be very difficult to show that they are *not* involved. The cautious mention of spontaneous GSH-isothiocyanate conjugation in more recent publications (Meyer *et al.*, 1995; Traka & Mithen, 2009) suggests that researchers are aware of this possibility. Yet, a thorough analysis of the relative importance of GST-mediated vs. spontaneous conjugation under physiological conditions has not been performed. For humans, this question may not assume great importance because the major interest in isothiocyanates lies in the anticarcinogenic properties of the non-conjugated compounds. For insect herbivores though, the extent of GST-mediated vs. spontaneous conjugation might make a difference as they likely consume isothiocyanates in higher quantities (compared to body weight) and their metabolism generally deviates from the vertebrate one. Ultimately, this could also mean that insect GSTs function through another mechanism, that intracellular GSH levels are different or that the overall metabolic strategies are profoundly dissimilar to those in vertebrates.

Role of GSTs and glutathione in isothiocyanate metabolism in generalist insect herbivores

The results described above all are derived from studies on human or rodent cells, so it cannot be assumed that the observed responses to isothiocyanates occur similarly in insects. It is known though that isothiocyanates, either derived from isothiocyanate-producing plants or administered in artificial diets, induce GST activity in lepidopteran herbivores (Yu, 1982; Wadleigh & Yu, 1988). However, this increase of GST activity is often based on assays with synthetic substrates, like 1-chloro-2,4-dinitrobenzene (CDNB), used because it forms a coloured product, rather than with the isothiocyanates themselves. Whether GSH conjugates with isothiocyanates are actually formed in insects, and whether isothiocyanate metabolism also occurs via the mercapturic acid pathway has so far not been investigated.

Since generalist locusts possess an inducible sulphatase that helps them avoid isothiocyanate formation (Falk & Gershenzon, 2007), similar strategies might also be found in other generalists. Furthermore, there could be a difference between extremely polyphagous generalists and those which show a feeding preference for brassicaceous plants, like for instance the cabbage looper *Trichoplusia ni*.

Another question to address would be that of the involvement of GSH in isothiocyanate metabolism, not only in terms of conjugation but also intracellular depletion. In human cancer cells this GSH depletion is substantial, but transient and it is eventually over-compensated by induction of GSH biosynthesis. In this way, the cells may not be exposed to negative effects by isothiocyanates for extended periods of time. Whether this is the same in insects is unknown. Generally, GSH availability in insects may be poor as plants often provide only low nitrogen. GSH depletion could hence be difficult to antagonise. From another perspective, insects may encounter much higher isothiocyanate concentrations than those which have been used in the experiments on human cancers cells. There, most of the effects were observed with low concentrations in the micro-molar range. An insect herbivore, entirely depending on plant material for nutrition, often completes its life cycle on the same plant and will thus frequently consume isothiocyanates if raised on a cruciferous plant. Additionally, in human consumption, myrosinases will mostly be inactivated before ingestion of plant material by boiling or other processing of food. Glucosinolate hydrolysis often mostly depends on the myrosinase activity of human enterobacteria and may thus be delayed, slowed down or different than when catalysed by plant myrosinases. Insects, in contrast, may be faced with an entirely different course of glucosinolate hydrolysis, as these herbivores always ingest plant tissue containing active myrosinases. Hence, they might be confronted with a more immediate, more aggressive and faster formation of isothiocyanates. All of these factors may influence how isothiocyanates affect lepidopteran herbivores.

In this thesis, many of the questions raised above were addressed. Chapter I deals with the question which metabolites generalist lepidopteran herbivores excrete after consumption of isothiocyanate-producing plant material. Does excretion depend on isothiocyanate structure? And is excretion of metabolites a common trait among different species of lepidopteran herbivores? Chapter II addresses the question whether GSTs are involved in the metabolism of isothiocyanates. Enzyme assays were conducted to determine the enzymatic activity of GSTs under different conditions. How much do GSTs contribute to the conjugation and deactivation of isothiocyanates? How much influence do other factors have, such as pH or substrate concentration? In Chapter III, the effects of isothiocyanates on caterpillar development were investigated. Are those effects dose-dependent? How do isothiocyanates affect GSH-levels in caterpillar guts? Do GSH-levels influence larval performance?

2 Metabolism of isothiocyanates in generalist lepidopteran herbivores by conjugation with glutathione

Katharina Schramm, Michael Reichelt, Jonathan Gershenzon, Ute Wittstock

Abstract

The defensive properties of the glucosinolate-myrosinase system in plants of the order Brassicales have been attributed to the formation of toxic isothiocyanates generated upon tissue damage. Lepidopteran herbivores specialised on brassicaceous plants have been shown to possess biochemical adaptations, preventing the formation of isothiocyanates. Yet, no such adaptational mechanisms are known for generalist lepidopterans which also occasionally feed on plants of the Brassicales. Analysis of the faeces of the generalist larvae of *Spodoptera littoralis*, which had fed on different Brassicaceae, revealed that this species forms glutathione conjugates of glucosinolate-derived isothiocyanates, whenever isothiocyanates are formed in the food plant. In the same way, the faeces of several other species of generalist caterpillars (*S. exigua*, *Mamestra brassicae*, *Trichoplusia ni* and *Helicoverpa armigera*) contain such glutathione conjugates of host plant-derived ITCs. The caterpillars of all species investigated are able to form glutathione conjugates from a large variety of isothiocyanate structures, suggesting detoxification via glutathione-S-transferase (GST) activity. Future investigations will explore the costs of this detoxification ability and determine whether glutathione-*S*-transferase activity is sufficient to cope with the range and quantity of isothiocyanates present in potential hosts.

2.1 Introduction

Plants produce a large diversity of secondary metabolites. Many of these compounds are toxic and serve as anti-herbivore defences (Fraenkel, 1959; Wittstock & Gershenzon, 2002; Hartmann, 2007). In order to use plants as a food source, phytophagous insects have to cope with these defences. In the course of evolution, many herbivores have become specialised to a family or a genus of plants with structurally similar defence compounds (Bernays & Chapman, 1994). This is thought to limit the range of potentially harmful secondary metabolites encountered by these herbivores, allowing them to develop detoxification or avoidance mechanisms specifically directed at the class of compounds present in their host plant. In contrast, generalist insect herbivores feed on a broad range of host plants from different families. Due to the diversity of plant chemical defences encountered, generalist herbivores are expected to have mechanisms to circumvent a large variety of different defence compounds (Brattsten, 1992).

One of the best studied plant defence systems is the glucosinolate-myrosinase system of the Brassicales, found in agriculturally important crops such as oilseed rape, cabbage and broccoli. Glucosinolates serve as defence against chewing insects after enzymatic activation by myrosinases which are stored in separate compartments in the intact plant tissue. When the tissue is damaged, these two components come into contact and the myrosinases hydrolyse the glucosinolates to form an array of bioactive compounds, such as isothiocyanates and nitriles, depending on the presence of so-called specifier proteins (Lambrix *et al.*, 2001; Burow *et al.*, 2009) (Fig. 1). Specifier proteins promote the production of nitriles, while in their absence isothiocyanates are formed (Fig. 1). Isothiocyanates are known to be toxic to a wide variety of organisms (Lichtenstein *et al.*, 1962; Seo & Tang, 1982; Buskov *et al.*, 2000; Tierens *et al.*, 2001; Wittstock *et al.*, 2003) including specialised feeders on glucosinolate-containing plants (Agrawal & Kurashige, 2003). Nitriles, on the other hand, appear to have lower direct toxicity (Lambrix *et al.*, 2001; Wittstock *et al.*, 2003; Burow *et al.*, 2006b; Zhang *et al.*, 2006) and instead serve to deter oviposition and attract herbivore enemies (de Vos *et al.*, 2008; Mumm *et al.*, 2008). Isothiocyanate toxicity, on the other hand, is comparable to that of commercial synthetic insecticides for lepidopteran insects (Wittstock *et al.*, 2003). Although the precise mode of action of isothiocyanates is yet unknown, it is believed that the toxicity of these compounds derives from the reaction of the isothiocyanate group (-NCS) with amino acid residues of proteins, such as the cleavage of disulfide bonds (Kawakishi & Kaneko, 1987).

Although generalist lepidopterans like the cabbage looper (*Trichoplusia ni*; Lepidoptera: Plusiinae) or the cabbage moth (*Mamestra brassicae*; Lepidoptera: Noctuidae) can also impose considerable agronomical and economical damage on glucosinolate-containing crops, studies on the detoxification of the glucosinolate-myrosinase system have focused mostly on specialist herbivores.

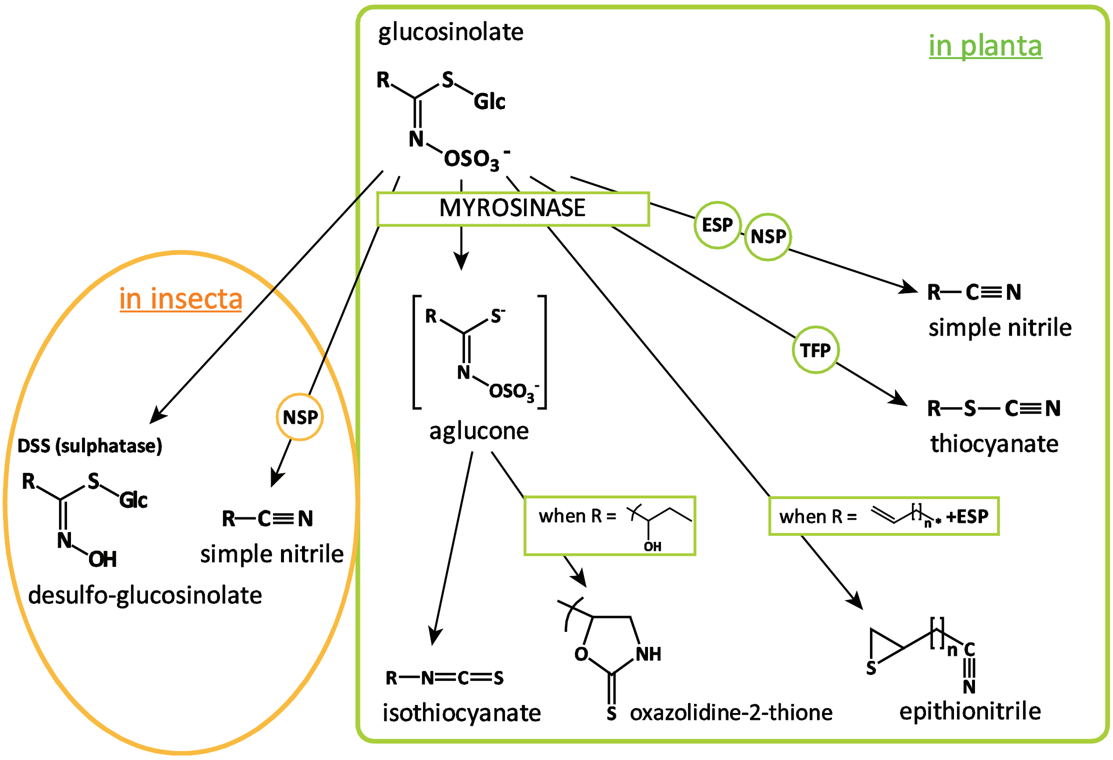

Fig. 1: Glucosinolate hydrolysis as it occurs in the leaves of *Arabidopsis thaliana* Col-0 and in insect herbivores feeding on plants of the Brassicaceae.

In the intact plant, glucosinolates and myrosinases are stored in separate compartments. Upon tissue damage, the two components come into contact and the myrosinases hydrolyse the glucosinolates to glucose and unstable aglucones which spontaneously rearrange to isothiocyanates. In the presence of plant epithiospecifier protein (ESP), glucosinolates with a terminal double bond in the side chain are hydrolysed to epithionitriles, and all other glucosinolates to simple nitriles. Nitriles are also formed in the presence of plant nitrile-specifying protein (NSP).

Lepidopteran larvae specialised on feeding on glucosinolate-containing plants possess special biochemical adaptations: larvae of *Plutella xylostella* and *Schistocerca gregaria* desulphate the glucosinolates, so those are no longer accepted as substrates by the myrosinases. The NSP of *Pieris rapae* caterpillars redirects hydrolysis towards simple nitriles.

Specialists on glucosinolate-containing plants like the cabbage white butterfly, *Pieris rapae* (Lepidoptera: Pieridae) or the diamondback moth *Plutella xylostella* (Lepidoptera: Plutellidae) circumvent the formation of toxic isothiocyanates with particular biochemical adaptations.

P. rapae larvae possess a gut-localized nitrile specifier protein (NSP) redirecting glucosinolate hydrolysis by plant myrosinases towards the less toxic nitriles which are then excreted with the faeces (Wittstock *et al.*, 2004) while *P. xylostella* makes use of a glucosinolate sulphatase (Ratzka *et al.*, 2002). Desulphated glucosinolates are no longer substrates for myrosinase-catalysed hydrolysis. Both of these adaptations prevent exposure to toxic isothiocyanates (Fig. 1). Yet, a search for NSP or sulphatase activity in generalist lepidopterans (including *Spodoptera littoralis*, *Heliothis virescens*, *Helicoverpa zea* (Lepidoptera: Noctuidae), and *T. ni*) did not detect these activities in gut extracts (Wheat *et al.*, 2007). The only generalist insect for which the mechanism of glucosinolate detoxification is known is not a lepidopteran but the generalist locust *Schistocerca gregaria* (Orthoptera: Acrididae). Like *P. xylostella*, it uses a sulphatase when it feeds on glucosinolate containing plants (Falk & Gershenzon, 2007).

In contrast to our limited knowledge on the formation and fate of glucosinolate-derived isothiocyanates in herbivorous insects, isothiocyanate metabolism has been widely studied in humans because of the anti-carcinogenic properties of certain isothiocyanates (Traka & Mithen, 2009). For mammals, isothiocyanates have been reported to be detoxified by enzymatic or non-enzymatic conjugation with glutathione (GSH) (Kassahun *et al.*, 1997; Al Janobi *et al.*, 2006); (Kolm *et al.*, 1995; Zhang *et al.*, 1995). General detoxification enzymes like glutathione-*S*-transferases (GSTs) are found in nearly all living organisms, including insects (Brattsten, 1992), and it has been suggested that also generalist lepidopteran larvae use GSTs to detoxify isothiocyanates (Yu, 1984; Yu, 1987; Wadleigh & Yu, 1988a; Wadleigh & Yu, 1988b). However, GSH conjugates of glucosinolate-derived isothiocyanates have not been identified in assays of these enzymes *in vitro* or in lepidopteran herbivores *in vivo*.

Here, we used liquid chromatography-mass spectrometry (LC-MS) to search for GSH conjugates in faeces of *Spodoptera littoralis* larvae that had ingested plant material with various profiles of glucosinolates and their hydrolysis products. We show that these larvae excrete GSH and cysteineglycine-conjugates (CysGly) of isothiocyanates derived from glucosinolates with various side chain structures. Additional feeding experiments with larvae of *S. exigua*, *T. ni*, *H. armigera* (Lepidoptera: Noctuidae) and *M. brassicae* demonstrate that the conjugation of isothiocyanates with GSH seems to be a common detoxification mechanism for glucosinolate-derived isothiocyanates in generalist lepidopteran herbivores.

Our findings are important for a better understanding of the mechanisms employed by generalist lepidopteran herbivores to cope with the glucosinolate-myrosinase system. They also provide important knowledge on how generalist lepidopteran herbivores interact with their host plants and how defence and counter-defence occur in plant-herbivore interactions.

2.2 Materials and Methods

2.2.1 Organisms and Chemicals

Arabidopsis thaliana was cultivated in a controlled environment growth chamber under short day conditions (10 h light, 14 h darkness) at 21 °C and 50-60 % relative humidity (RH). The different *A. thaliana* genotypes used are summarized in Table 1. *Brassica nigra* and *Eruca sativa* were grown in a controlled greenhouse at 21 °C with a rhythm of 12 h light and 12 h darkness at 50-60 % RH.

Caterpillars of *S. littoralis* (Egyptian Cotton Leafworm; Boisduval) and *S. exigua* (Beet Armyworm; Hübner), both obtained from Syngenta (Maintal, Germany), were reared on an artificial diet based on white beans (modified after Bergomaz, 1986) in the laboratory at 20 °C and 22 °C, respectively, under natural light. Larvae of *Helicoverpa armigera* (Cotton Bollworm, Toowoomba strain; Hübner) were reared on a pinto bean diet (Perkins et al., 1973) in a controlled-environment room at 26 °C, 16:8 (light:darkness) photoperiod, and 60 % RH until used for experiments. Caterpillars of *Mamestra brassicae* (Cabbage Moth; Linnaeus), provided by J. Harvey (NIOO Wageningen, The Netherlands) and caterpillars of *Trichoplusia ni* (Cabbage Looper; Hübner), purchased from Benzon Research (Carlisle, USA) were reared on a diet based on wheat germ (Burton, 1969) at room temperature under natural light conditions.

L-glutathione (GSH) and isothiocyanates were obtained from Sigma-Aldrich (Munich, Germany) with the exception of 3-isothiocyanato-1-propene (allylisothiocyanate) which was purchased from Fluka (Buchs, Switzerland).

2.2.2 Chemical syntheses

GSH conjugates of isothiocyanates were chemically synthesised according to Kassahun et al. (1997) with slight modifications. GSH (5 mg) was dissolved in 0.5 ml 50 % (v/v) ethanol (pH 7.5) and mixed with 1 ml of 0.5 % (v/v) isothiocyanate solution in ethanol. The mixture was agitated under nitrogen on a vortex shaker overnight. The identity of the reaction products was confirmed by LC-MS based on the *m/z* of fragments observed in MS^2 and MS^3 spectra.

2.2.3 Analysis of aqueous faeces extracts and products of enzyme assays by LC-MS

All HPLC analyses were carried out on Agilent 1100 series equipment (Agilent Technologies, Böblingen, Germany). The chromatograph was coupled to an Esquire 6000 ESI-Ion Trap mass spectrometer (Bruker Daltonics, Bremen, Germany) operated in a positive mode in the range *m/z* 50-1000 with skimmer voltage, -40 eV; capillary exit voltage, -146.7 eV; capillary voltage, 4000 V; nebulizer pressure, 35 psi; drying gas, 11 l*min^{-1}; and gas temperature, 330 °C. Separation was accomplished using a EC 250/4.6 Nucleodur Sphinx RP column (25 cm x 4.6 mm, 5 µm, Macherey-Nagel, Düren, Germany) with a gradient of 0.2 % (v/v) formic acid (solvent A) and acetonitrile (solvent B). For faeces extracts, separation was accomplished at a flow rate of 0.8 ml min^{-1} at 25 °C as follows: 0-30 % B (30 min) 30-100 % B (0.1 min), a 3 min hold at 100 % B, 100-0 % B (0.1 min) and a 3 min hold at 0 % B. Enzyme assays were analyzed on the same system using the following gradient at 1 ml min^{-1}: 5-38 % B (11 min), 38-100 % B (0.1 min), 100 % B for 3 min hold, 100-5 % B (0.1 min) and a 5 min hold at 5 % B. The eluate was split off in a ratio 4:1 before reaching the mass spectrometer. Chromatograms were analysed with the DataAnalysis software from Bruker Daltonics.

2.2.4 Analysis of glucosinolate hydrolysis products in leaf homogenates by gas chromatography (GC)

Fresh leaf material (300 to 600 mg) was crushed in 300 µl H_2O containing 0.05 µmol of phenyl cyanide in methanol as internal standard. After addition of 750 µl of dichloromethane and thorough mixing, the two phases were separated by centrifugation at 3030 x*g* for 10 min. The organic phase was removed, and the aqueous phase was re-extracted with another 750 µl of dichloromethane. The organic extracts were pooled, dried over Na_2SO_4, evaporated under nitrogen to ~200 µl, and 1 µl was analyzed. Separation, identification and quantification of glucosinolate hydrolysis products was done by GC-mass spectrometry (MS) or -flame ionisation detection (FID) as described previously (Lambrix *et al.*, 2001; Burow *et al.*, 2006b) on an Agilent 6890 series gas chromatograph coupled with an Agilent 6973 Network series mass spectrometer or a flame ionisation detector. Separation was achieved on an SLB-5ms silica capillary column (30 m x 0.25 mm x 0.25 µm film; Supelco, Bellefonte, USA) using splitless injection at 200 °C, and the following temperature program: 40 °C for 3 min, a 10 °C*min^{-1} ramp to 250 °C, a 60 °C*min^{-1} ramp to 300 °C, hold for 3 min.

2.2.5 Glucosinolate analysis

Glucosinolates were analysed after conversion to desulfo-glucosinolates. For this, 300-600 mg of fresh leaf material were ground with a micropestle in 1-2 ml of 80 % methanol containing 0.05 µmol *p*-hydroxybenzylglucosinolate as an internal standard. After centrifugation (15 min at 3030 x*g*), the supernatant was applied to a column containing 1 ml DEAE-sephadex A25 equilibrated with water. The column was washed consecutively with 2 x 1 ml 67 % (aqueous) methanol and 1 ml water before the addition of 1 ml 0.02 mM MES buffer, pH 5.2. After this 50 µl of *Helix pomatia* sulphatase (solution prepared as described in Graser et al. 2001) were added and left to incubate overnight. Desulfo-glucosinolates were eluted with 0.8 ml 60 % methanol and 0.8 ml water and dried at 45 °C under a stream of nitrogen. After resuspension in 0.4 ml water the samples were analyzed by high performance liquid chromatography (HPLC) on an Agilent HP1100 Series instrument, equipped with a C-18 reversed phase column (LiChrospher RP18ec, 250×4.6 mm, 5 µm particle size) by using a water (solvent A) - acetonitrile (solvent B) gradient at a flow rate of 1 ml*min^{-1} at 25 °C (injection volume 40 µl). The gradient was as follows: 1.5% B (1 min), 1.5-5 % B (5 min), 5-7 % B (2 min), 7-21 % (10 min), 21-29 % (5 min), 29-43 % (7 min), 43-100 % (0.5 min), 100 % B (2.5 min), 100-1.5 % B (0.1 min), and 1.5 % B (4.9 min). The eluent was monitored by diode array detection between 190 and 360 nm (2 nm interval).

Desulfo-glucosinolates were identified based on comparison of retention times and UV absorption spectra with those of known standards (Reichelt *et al.*, 2002). Results are given as µmol (*g dry weight^{-1}) calculated from the peak areas at 229 nm relative to the peak area of the internal standard, using the relative response factors 2.0 for aliphatic and 0.5 for indole glucosinolates (Burow *et al.*, 2006b), respectively.

2.2.6 Feeding experiments and preparation of faeces extracts

Third instar caterpillars of all species were switched from their respective artificial diets onto detached leaves of different plant species (*A. thaliana* Col-0 wildtype, gsm1-1 and 35S::ESP plants, *Brassica nigra* and *Eruca sativa*) mounted in closed plastic containers (one larva per container) and held in a controlled environment chamber at 21 °C with a 16 h light period. Faeces were collected daily for a period of five days, pooled and stored in 1 ml of 100 mM citrate buffer, pH 3.5, at 4 °C. Of the collected faeces, 1 to 1.5 g (fresh weight) were extracted three times in a total of 4 ml 100 mM citrate buffer, pH 3.5, by vortexing. After centrifugation at 4,300 x*g* and 4 °C for 10 min, the supernatants were pooled and extracted three times with a total of 4 ml dichloromethane. Phases were separated by centrifugation at 4,200 x*g* and 4 °C for 10 min. The aqueous phase was filtered through a 45 µm sterile filter, evaporated under a nitrogen stream at room temperature to approximately 1.5 ml and subsequently analysed by LC-MS for GSH conjugates.

For the comparison of conjugate formation after ingestion of *A. thaliana* Col-0 and CaMV35S:ESP plants, faeces (total of 180-300 mg fresh weight of faeces per larva) were collected individually from 10 caterpillars per plant genotype and pooled over five days. Extraction of faeces from each individual caterpillar followed the same protocol as above but with only 2 ml of 100 mM citrate buffer and 2 ml of dichloromethane. As an internal standard, 40 µl of a 0.1 mM solution of butylisothiocyanate-GSH conjugate was added to each sample at the beginning of the extraction procedure. Results are given as nmol*g^{-1} fresh weight of faeces calculated from the peak areas of the compound peaks at 254 nm relative to the peak area of the internal standard and comprise the sum of both the GSH- and GlyCys-conjugates of 4-methylsulfinlybutyl isothiocyanate.

Table 1: Conversion of glucosinolate-derived hydrolysis products from various plants to GSH and CysGly conjugates by generalist caterpillars. The main glucosinolate and hydrolysis product of each species was determined by homogenization of leaves of 5 week old plants. When conjugate was found in the faeces of *Spodoptera littoralis*, *S. exigua*, *Helicoverpa armigera*, *Mamestra brassicae* and *Trichoplusia ni*, *m/z* is listed. Conjugates were found for all isothiocyanate hydrolysis products, but not for the nitrile. The identities of the conjugates were confirmed by comparison with spectral data of standards obtained by chemical synthesis (except for the conjugate of 3-methylthiobutyl isothiocyanate for which no commercial standard was available).

Plant	**main glucosinolate**	**main hydrolysis product type**	***m/z* GSH conjugate**	***m/z* CysGly conjugate**
Arabidopsis thaliana, Col-0	4-methylsulfinylbutyl	isothiocyanate	485	356
A. thaliana CaMV35S::ESP, line 37.3 (Burow *et al.*, 2006a)	4-methylsulfinylbutyl	nitrile	--	--
A. thaliana CaMV35S::Cyp79 A2 (Wittstock & Halkier, 2000)	benzyl	isothiocyanate	457	328
A. thaliana mutant TU1/gsm1-1 (Haughn *et al.*, 1991; Kroymann *et al.*, 2001)	3-methylsulfinylpropyl	isothiocyanate	471	342
Brassica nigra	allyl	isothiocyanate	407	278
Eruca sativa	4-methylthiobutyl	isothiocyanate	469	340

2.3 Results

2.3.1 The GSH conjugate of 4-methylsulfinylbutyl isothiocyanate (4msob-ITC) is excreted in *Spodoptera littoralis* faeces after feeding on *A. thaliana* Col-0

In order to test if ingestion of glucosinolate-containing plant material by *S. littoralis* larvae results in the excretion of GSH conjugates of the corresponding isothiocyanates, we analyzed extracts of faeces from caterpillars that had fed on rosette leaves of *Arabidopsis thaliana* Col-0 by LC-MS. The major glucosinolate in rosette leaves of *A. thaliana* Col-0 is 4-methylsulfinylbutylglucosinolate (Brown *et al.*, 2003). During hydrolysis, this glucosinolate is broken down mainly to the corresponding isothiocyanate (Lambrix *et al.*, 2001). The LC-MS chromatogram displayed a peak with a retention time of 18.3 min representing a compound with a $m/z = 485$. This compound was identified as the GSH conjugate of 4msob-ITC based on the comparison of the mass spectrum, MS/MS spectra and retention time with those of a standard synthesized after Kassahun et al. (1997) (Fig. 2). A compound eluting at 15.1 min with a $m/z = 356$ was identified as a metabolite of the GSH conjugate comprising the 4msob-ITC moiety and a cysteine-glycine dipeptide (CysGly conjugate). We did not detect the N-acetylcysteine conjugate ($m/z = 341$) of 4msob-ITC, which is a major metabolite of this isothiocyanate in mammals (Kassahun *et al.*, 1997; Al Janobi *et al.*, 2006). Other possible glucosinolate detoxification products, such as desulfo-glucosinolates or nitriles, were also not detected. Thus 4msob-ITC, a glucosinolate hydrolysis product formed after damage to *A. thaliana* Col-0 rosette leaves, is metabolised to GSH and CysGly conjugates in larvae of *S. littoralis*. In addition to the conjugates, we detected varying amounts of 4msob-ITC.

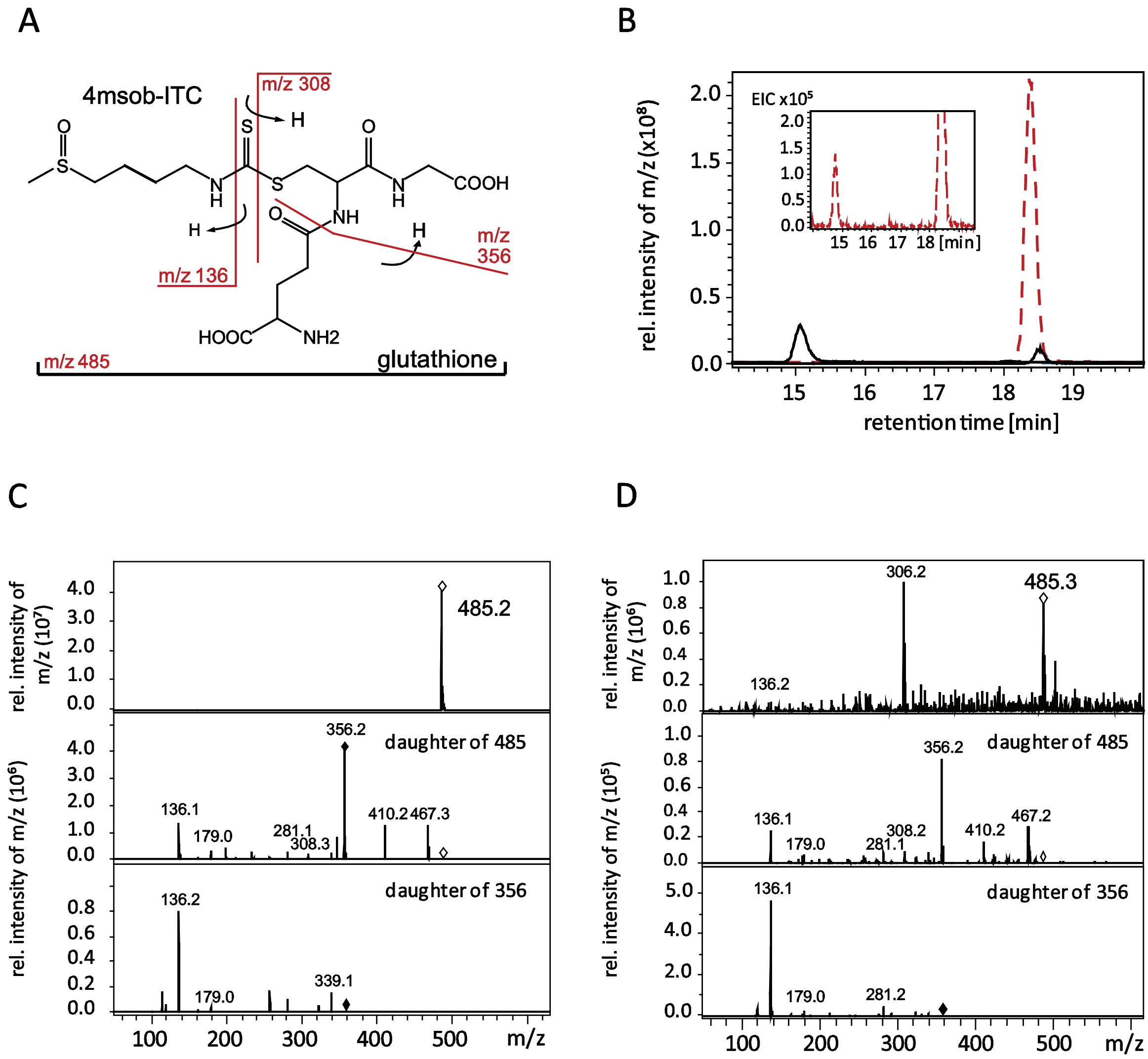

Fig. 2: Presence of the GSH and CysGly conjugates of 4-methylsulfinylbutyl isothiocyanate (4msob-ITC) in faeces of *S. littoralis* after ingestion of *A. thaliana* Col-0 wildtype leaves.

(A) Chemical structure and MS fragmentation pattern (m/z in positive ion mode) of the GSH conjugate of 4msob-ITC.

(B) LC-MS chromatograms (extracted ion chromatogram at *m/z* = 485 and *m/z* = 356) of an extract of *S. littoralis* faeces, obtained after feeding on *A. thaliana* Col-0 wildtype plants (solid line), and of synthetic 4msob-ITC-conjugate standard (dashed line). The compound eluting at 18.1 min was identified as the GSH conjugate of 4msob-ITC (m/z 485), the compound eluting at 15.3 min as the corresponding CysGly conjugate (m/z 356). In the insert, the chromatogram of the standard is redrawn with a different scale to show the presence of a peak at 15.3 min.

(C) MS/MS spectra of the synthetic GSH conjugate.

(D) MS/MS spectra of the GSH conjugate identified in *S. littoralis* faeces.

To further test the association of dietary isothiocyanate with GSH conjugate formation in *S. littoralis*, larvae were fed transgenic *A. thaliana* plants (35S::ESP plants) that produce a lower proportion of isothiocyanates and a higher proportion of simple nitriles upon tissue disruption than wild-type plants due to overexpression of the epithiospecifier protein (ESP) (Burow *et al.*, 2006b) (Table 2). In faeces of *S. littoralis* caterpillars feeding on leaves of 35S::ESP plants, the content of GSH and CysGly conjugates was reduced by more than 50 % in comparison to faeces from caterpillars feeding on wildtype plants (student's t-test, p< 0.001; Fig. 3). GSH or CysGly conjugates of the nitrile derived from 4-methylsulfinylbutylglucosinolate were not detected.

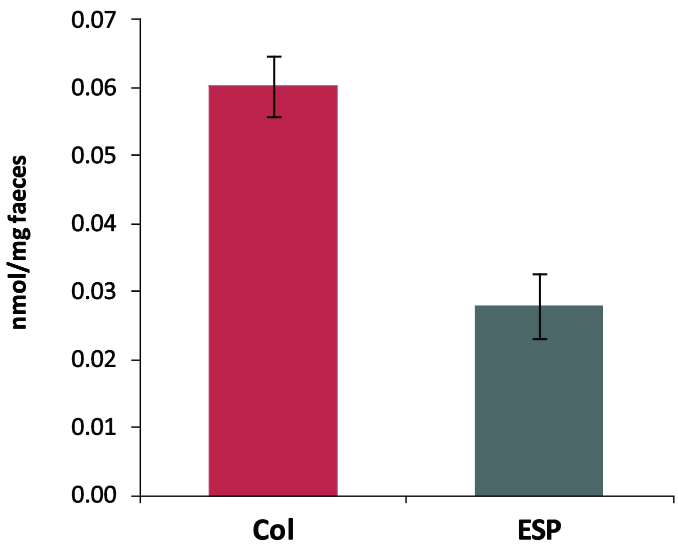

Fig. 3: Concentration of GSH and CysGly conjugates of 4msob-ITC in the faeces of *S. littoralis* after feeding on leaves of *A. thaliana* Col-0 wildtype or transgenic plants overexpressing ESP (35S::ESP).

Caterpillars were allowed to feed on either wildtype or 35S::ESP plants for 24-48 h. Faeces were collected daily. Faeces extracts in 100 mM citrate buffer, pH 3.5, were subjected to LC-MS analysis. Faeces of caterpillars feeding on Col-0 wildtype plants contain significantly more conjugates than faeces of caterpillars feeding on 35S::ESP plants (n=20; student's t-test, $t = 4.969$; $\Delta f = 18$; $p < 0.001$).

Table 2: Comparison of the levels of 4-methylsulfinylbutylglucosinolate (4msob), the predominant glucosinolate in rosette leaves of *A. thaliana*, and the respective isothiocyanate and nitrile hydrolysis products formed upon homogenisation in leaves of the Col-0 WT and the 35S::ESP line 37.3 (n = 6). Values of the isothiocyanate and the nitrile are given as percentages of the total amount of hydrolysis products from 4-methylsulfinylbutylglucosinolate (isothiocyanate + nitrile) as means ± SD (n=6).

	4msob glucosinolate [µmol/g FW] ± SD	4msob-ITC [%] ± SD	4msob-nitrile [%] ± SD
Col-0 wild type	1.10 ± 0.17	70.4 ± 5.1	29.6 ± 8.2
35S::ESP, line 37.3	1.08 ± 0.18	10.7 ± 13.1	89.3 ± 17.3
student's t-test	$t = 0.27$; $\Delta f = 11$; $p = 0.79$	$t = 7.116$; $\Delta f = 4$; $p = 0.002$	$t = -7.116$; $\Delta f = 4$; $p = 0.002$

2.3.2 Isothiocyanates of varying structure are metabolized to GSH conjugates in *S. littoralis*

To investigate if conjugation with GSH is a metabolic pathway for isothiocyanates derived from different glucosinolate precursors in *S. littoralis*, we conducted feeding tests with a range of plants (*A. thaliana* Col-0 wildtype, gsm1-1 and 35S::ESP plants, *Brassica nigra* and *Eruca sativa*), containing glucosinolates with different aliphatic or aromatic side chains (Table 1). For the major glucosinolate of each species or genotype, both GSH and CysGly conjugates of the corresponding isothiocyanate were detected by LC-MS analyses of aqueous faeces extracts (Table 1). The identity of these conjugates was determined as mentioned above by comparison of mass spectra and retention times with those of standards made by chemical syntheses. Neither *N*-acetylcysteine conjugates of any of these isothiocyanates nor desulfo-glucosinolates were detected in the faeces. The respective isothiocyanates were detectable in varying but small quantities in the organic faeces extracts. Nitriles were not detected at all (except for the reversed situation, in faeces from ESP-fed caterpillars).

2.3.3 GSH-isothiocyanate conjugates are found in faeces of different species of generalist caterpillars

To see whether the metabolism of glucosinolate-derived isothiocyanates to GSH conjugates is a metabolic conversion generally found in polyphagous caterpillars, other generalist lepidopteran herbivores, namely larvae of *S. exigua*, *Trichoplusia ni*, *Helicoverpa armigera* and *Mamestra brassicae*, were allowed to feed on leaves of *A. thaliana* Col-0 plants. The faeces of all species tested contained the GSH conjugate of 4msob-ITC (m/z 485, Fig. 4), as well as the corresponding CysGly conjugate (m/z 356; data not shown).

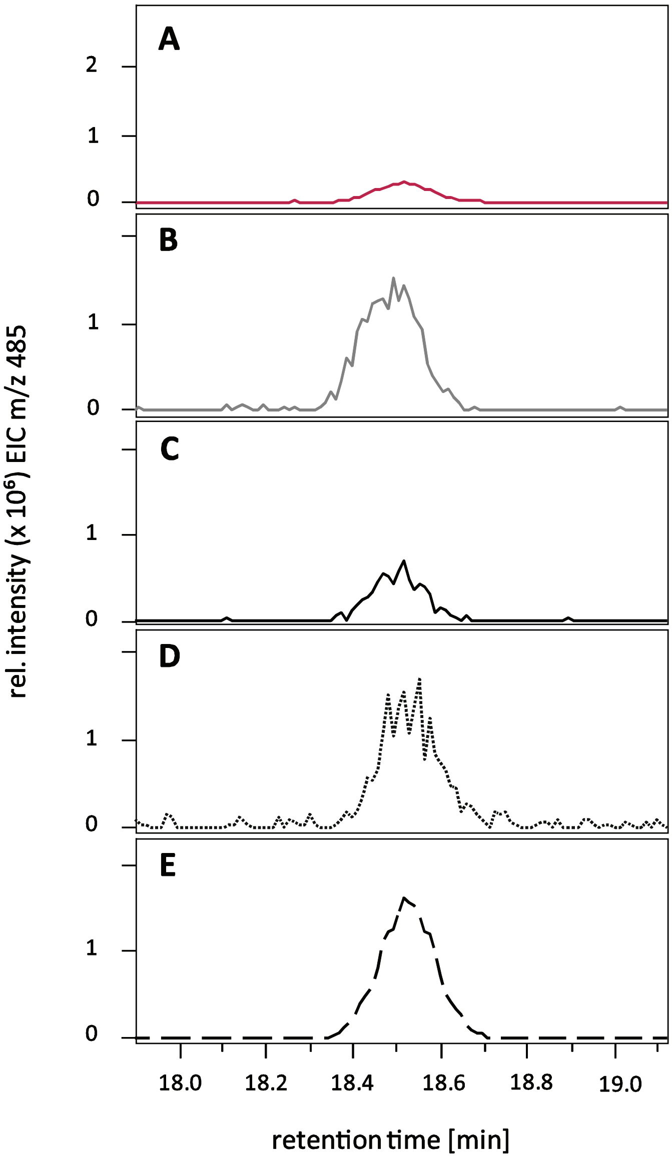

Fig. 4: Presence of the GSH conjugate with 4msob-ITC in faeces of different species of generalist caterpillars after feeding on *A. thaliana* Col-0 wildtype plants.

Extracted ion chromatograms (EIC) of m/z 485 in faeces extracts of *Spodoptera littoralis* (A), *Helicoverpa armigera* (B), *Trichoplusia ni* (C), *Mamestra brassicae* (D), and *Spodoptera exigua* (E).

2.4 Discussion

Generalist lepidopteran larvae feed on a broad variety of host plants where they encounter an equally broad range of plant secondary defence compounds. When feeding on plants of the Brassicaceae, these larvae frequently have to deal with toxic isothiocyanates released from glucosinolates upon tissue damage. While caterpillars specialised on these plants possess biochemical adaptations that prevent the formation of isothiocyanates (Ratzka *et al.*, 2002; Wittstock *et al.*, 2004), such mechanisms seem to be absent from generalist caterpillars (Wheat *et al.*, 2007). Thus, generalist caterpillars are likely exposed to the toxic isothiocyanates. Here, we provide evidence that a range of generalist caterpillars with different feeding preferences for glucosinolate-containing plants metabolise at least a proportion of the ingested isothiocyanates by conjugation with glutathione (GSH).

GSH and cysteineglycine (CysGly)-isothiocyanate conjugates were identified in the faeces of *S. littoralis*, *S. exigua*, *H. armigera*, *T. ni*, and *M. brassicae* after the larvae had fed on glucosinolate-containing plants (Figs. 2 & 4; Table 1). In contrast to most previous studies (Wadleigh & Yu, 1988a; Yu, 1989; Yu, 2002b) on isothiocyanate detoxification in insects, we used intact plants instead of artificial diets spiked with isothiocyanates in our feeding tests. This enabled us to study the metabolism of glucosinolate-derived isothiocyanates released by natural glucosinolate hydrolysis. As we have detected conjugates of glucosinolate-derived isothiocyanates in larval faeces and as the structure of the isothiocyanate moiety varied depending on the structure of the ingested glucosinolate, we can conclude that isothiocyanates, released upon ingestion of plant material by generalist caterpillars, can be metabolised to GSH- and CysGly conjugates.

GSH- and CysGly conjugates were formed from a variety of different isothiocyanate structures that were in turn derived from a range of glucosinolate types (Table 1). Among the isothiocyanates tested were methylsulfinylalkyl, methylthioalkyl, alkenyl and aromatic compounds all of which were found to be conjugated with GSH. For *S. littoralis*, we demonstrated that the amount of conjugate formed depends on the amount of isothiocyanate available in the diet (Table 2) as feeding on transgenic *A. thaliana* with reduced isothiocyanate formation but increased nitrile formation (35S::ESP plants; (Burow *et al.*, 2006b) resulted in the excretion of significantly less GSH conjugate of 4-methylsulfinylbutyl isothiocyanate than feeding on Col-0 wildtype plants (Fig. 3). GSH and GlyCys conjugates with nitriles were not detected, even though these compounds would be expected to be detectable under these LC-MS conditions.

The absence of conjugates with nitriles is consistent with the fact that the nitrile moiety has a much lower reactivity than the isothiocyanate moiety in conjugation with glutathione. Nitriles are most likely metabolised in a different way than isothiocyanates, or are simply excreted in the faeces like in *P. rapae* (Wittstock et al., 2004).

While isothiocyanates are metabolised to *N*-acetyl cysteine conjugates in humans via the mercapturic acid pathway, no products other than the GSH and the GlyCys conjugates were detected in faeces of the caterpillar species tested. Lepidopteran larvae may lack the enzymes for this conjugation found in humans, or the reaction may be hampered by the high pH commonly found in the guts of lepidopteran larvae (Berenbaum, 1980). Our detection of GSH conjugates of isothiocyanates in lepidopteran faeces strongly suggests that these, like other types of GSH conjugates, are products of enzymatic catalysis mediated by glutathione-*S*-transferases. In humans (Kolm *et al.*, 1995; Zhang *et al.*, 1995) and cyanobacteria (Wiktelius & Stenberg, 2007), glutathione-S-transferase catalysis of isothiocyanate-GSH conjugates has been reported. However, studies using isothiocyanates as substrates for insect glutathione-*S*-transferases are scarce (Wadleigh & Yu, 1988a), and a participation of enzymes in the detoxification process has not yet been shown under physiological conditions.

Although we can conclude that there are lower amounts of isothiocyanate conjugates when isothiocyanate production is reduced in favour of nitrile production (Fig. 3), we cannot determine whether or not conjugation with GSH is the main (and possibly exclusive) pathway of isothiocyanate metabolism in *S. littoralis*. For this determination, isothiocyanate intake would have to be accurately measured. Such measurements have been carried out in humans as part of research to investigate the anti-carcinogenic properties of isothiocyanates (Egner *et al.*, 2008; Traka & Mithen, 2009). In humans, the main pathway of isothiocyanate metabolism includes rapid absorption into the enterocytes, conjugation with GSH, export into blood circulation, and subsequent conversion to N-acetyl cysteine conjugates which are excreted in the urine (Al Janobi *et al.*, 2006; Traka & Mithen, 2009). Administration of defined concentrations of isothiocyanates to caterpillars or feeding radioactively labelled glucosinolates whose metabolic fate can be traced would help resolve the importance of GSH conjugation as a route for isothiocyanate metabolism in *S. littoralis*.

Our study also provides evidence that isothiocyanate metabolism by conjugation with GSH may be widespread in generalist lepidopteran herbivores regardless of their feeding preferences (Table 1; Fig. 4). The two *Spodoptera* species studied do not naturally use glucosinolate-containing plants as hosts, although they can complete development on them. On the other hand, *H. armigera* is a broad generalist whose early larval stages have been reported to feed on plants of the Brassicaceae without showing a clear preference for them.

In contrast, *M. brassicae* and *T. ni* readily and frequently feed on plants of the Brassicaceae in nature and are serious pests on various *brassicaceous* crops (Carter, 1984; Finch & Thompson, 1992; Capinera, 2001). A widespread occurrence of GSH-mediated metabolism of isothiocyanates in Lepidoptera would be in agreement with this strategy being common to a broad range of living organisms, including cyanobacteria (Wiktelius & Stenberg, 2007). Yet, a possible disadvantage of GSH-mediated detoxification of isothiocyanates is its metabolic cost. For every mole of isothiocyanate detoxified, generalist caterpillars lose one mole of glutathione, containing three amino acids which may be in short supply. Costs may be especially high when species like *T. ni* or *M. brassicae* feed continuously on glucosinolate-containing plants throughout larval development. Therefore, it might be expected that generalist lepidopteran larvae employ additional, less costly mechanisms to cope with the glucosinolate-myrosinase system. Future investigations are planned to search for other mechanisms of glucosinolate and isothiocyanate metabolism in lepidopterans and to determine whether the conjugation process in caterpillars is catalysed by an enzymatic activity (Wadleigh & Yu, 1988a; Yu, 1989; Yu, 1995; Yu, 2002a) as has been shown for rats (Kassahun *et al.*, 1997), humans (Kolm *et al.*, 1995) and cyanobacteria (Wiktelius & Stenberg, 2007).

3 Detoxification of isothiocyanates in *Spodoptera littoralis* by conjugation with glutathione – a non-enzymatic reaction?

Katharina Schramm, Ute Wittstock, Jonathan Gershenzon

Abstract

Isothiocyanates are a frequent hydrolysis product from cruciferous plants and play an important role in human nutrition, because they make brassicaceous vegetables palatable and due to their anti-carcinogenic properties. In mammals, isothiocyanates are known to be metabolised via the mercapturic acid pathway. In the first step, they are conjugated with glutathione (GSH), a reaction which can be catalysed by mammalian glutathione-*S*-transferases (GSTs) *in vitro*. Whether GSTs perform this reaction at significant rates *in vivo* is uncertain. When feeding on crucifers, generalist insect herbivores may get in contact with isothiocyanates, yet little is known on how they deal with these noxious compounds. Recent publications have suggested that in generalist lepidopteran larvae, like in mammals, GSTs catalyse the conjugation of isothiocyanates with GSH.

Here, active native GSTs were purified from midguts of the generalist lepidopteran caterpillars of *Spodoptera littoralis*. We showed that under certain conditions these GSTs are capable of catalysing the conjugation reaction of GSH with isothiocyanates of different structures. Yet, in *in vitro* assays using physiological conditions simulating the environment of possible *in vivo* conjugation locations, GST activity was not detectable. Although GSTs probably play a variety of roles in *S. littoralis*, our results do not support a major role for GSTs in isothiocyanate metabolism.

3.1 Introduction

Plants produce a large diversity of secondary metabolites, many of which serve as anti-herbivore defences (Fraenkel, 1959; Wittstock & Gershenzon, 2002; Hartmann, 2007). In order to use plants as nutrient sources, phytophagous insects have to cope with these defences. In Lepidopterans, the larvae of many species have specialised to feed on only a few plant species (Brattsten, 1992). While this adaptation limits the host range of these species, it also reduces the variety of plant defence compounds they have to deal with. Specialist herbivores cope with specific defence compounds by becoming insensitive to the toxin (Holzinger & Wink, 1996), rapid excretion (Cohen *et al.*, 1992), sequestration (e.g. Muller & Wittstock, 2005) or protecting themselves in some other way (Ratzka *et al.*, 2002; Wittstock *et al.*, 2004). Polyphagous herbivores, on the other hand, usually feed on a broad range of plant species and thus encounter a plethora of metabolites capable of defending the plants against enemies. As a consequence, generalists need to have strategies for dealing with this diversity, such as avoidance of high concentrations of toxins, rapid excretion or general detoxification enzymes.

Plants of the order Brassicales defend themselves against chewing insects predominantly with the glucosinolate-myrosinase system. The defence compounds derived from this system pose special challenges for herbivorous attackers. Generated only when plant tissue is damaged, the outcome of myrosinase-driven hydrolysis differs depending on glucosinolate composition, myrosinase activity and the presence of specifier proteins and certain co-factors. Different hydrolysis products are of differing toxicity, with isothiocyanates believed to be most deleterious to insects. Their toxicity is comparable to that of commercial synthetic insecticides (Lichtenstein *et al.*, 1962). Isothiocyanates are believed to confer their toxicity by the highly reactive isothiocyanate moiety which *in vitro* reacts with amino acid residues of proteins and cleaves disulfide bonds (Kawakishi & Kaneko, 1987).

Generalist herbivores feeding on isothiocyanate-producing plants should therefore have a means of protecting themselves against the deleterious effects of these compounds. While little is known about how insects accomplish this, isothiocyanate metabolism has been studied extensively in humans because isothiocyanates have been suggested to possess anticancer activity. Here, diet-derived isothiocyanates are detoxified and excreted via the mercapturic acid pathway starting off with the conjugation of isothiocyanates to glutathione (GSH) (Habig *et al.*, 1974) (Fig. 1). A specific class of detoxification enzymes, widespread in animals and plants, has been shown to be capable of catalysing this step in different organisms such as plants, humans and cyanobacteria: the glutathione-*S*-transferases (GSTs) (Kolm *et al.*, 1995; Meyer *et al.*, 1995; Zhang *et al.*, 1995; Zhang, 2001; Nutricati *et al.*, 2006; Wiktelius & Stenberg, 2007).

Nonetheless, some of these studies also mention high non-enzymatic conjugation rates between isothiocyanates and GSH, leaving the contribution of GSTs to this process questionable. GSTs are well-known for their role in conjugating GSH with toxic xenobiotics, thereby detoxifying them (Ranson & Hemingway, 2005; Josephy & Mannervik, 2006; Traka & Mithen, 2009). Yet, many plant and animal GSTs perform a broad range of reactions unrelated to xenobiotic metabolism (Hayes *et al.*, 2005; Allocati *et al.*, 2009; Dabrowska *et al.*, 2009).

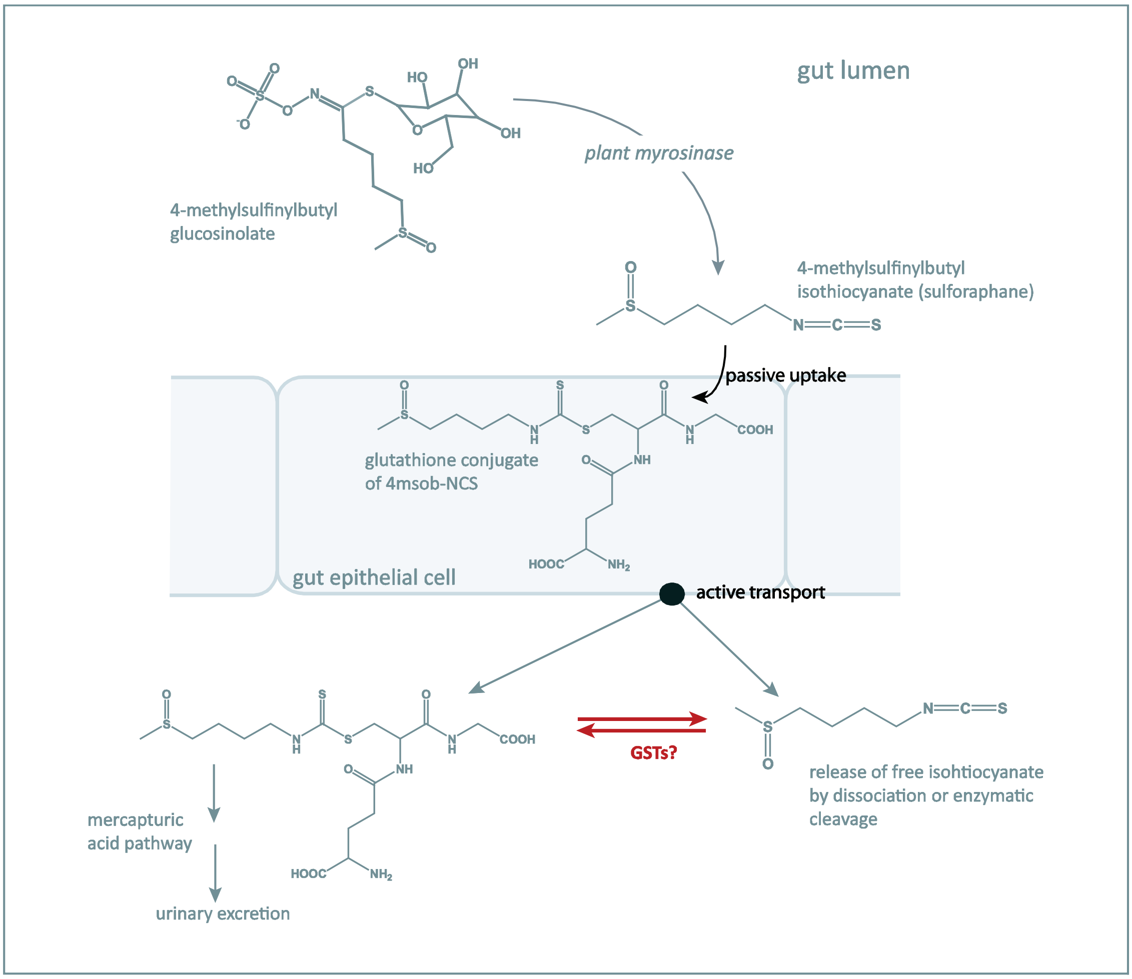

Fig. 1: Proposed route of isothiocyanate metabolism in humans (after Traka & Mithen, 2009).

Intact glucosinolates are ingested and hydrolysed by gut microbial myrosinases or by plant myrosinases during ingestion to form isothiocyanates. Isothiocyanates are passively taken up by gut epithelial cells where they are conjugated immediately with GSH. This conjugation may occur spontaneously or with catalytic help from GSTs. GSH-isothiocyanate conjugates are actively excreted from the cells and subsequently renally excreted via the mercapturic acid pathway. Alternatively, once removed from the cells, isothiocyanates are released from the conjugate and may exert effects as anti-carcinogenics.

Glutathione (L-γ-glutamyl-L-cysteinylglycine) is the most abundant non-protein thiol in mammalian cells. As a strong nucleophile, GSH can spontaneously react with many electrophiles (Ketterer, 1982; Ketterer *et al.*, 1983; Traka & Mithen, 2009) even without the catalysis of GSTs. Thus in mammalian cells, the high intracellular levels of GSH alone may constitute an important line of defence against reactive electrophiles, such as isothiocyanates, and oxidative stress (Griffith, 1999; Dickinson & Forman, 2002). Yet, the evidence for enzymatic vs. non-enzymatic conjugation of isothiocyanates with GSH is equivocal. The intracellular environment provides favourable conditions for a fast, non-enzymatic conjugation of GSH with electrophiles. GSH concentrations normally are high (2-10 mM in mammalian cells; Josephy & Mannervik, 2006) compared to most other metabolites, and at physiological pH spontaneous reaction of GSH and isothiocyanates is extensive (Kolm *et al.*, 1995). But GSTs are often also present in high concentrations (Ketterer *et al.*, 1983; Josephy & Mannervik, 2006) which would support the occurrence of enzymatic conjugation. *In vitro* studies showed that GSTs accept isothiocyanates as substrates, yet the very same studies clearly show that *in vitro* isothiocyanates react spontaneously with GSH (Kolm *et al.*, 1995; Meyer *et al.*, 1995; Zhang *et al.*, 1995; Wiktelius & Stenberg, 2007). According to recent publications, both spontaneous and enzyme-driven conjugation of isothiocyanates with GSH provides the first substrates of the mercapturic acid pathway in humans (Ketterer *et al.*, 1983; Zhang *et al.*, 1995; Lo *et al.*, 2007; Traka & Mithen, 2009). In summary, it is not certain whether GSTs actually participate in GSH-conjugation of isothiocyanates in humans or any other organism investigated.

Less is known about isothiocyanate metabolism in insects, with most research having been conducted on generalist lepidopteran larvae. Several studies have demonstrated that isothiocyanates induce GST activity in different species of generalist herbivorous caterpillars (Yu, 1982; Yu, 1983; Wadleigh & Yu, 1988a). When soluble protein fractions from herbivorous caterpillars were assayed using GSH and isothiocyanates as substrates, consumption of GSH was demonstrated. Based on these results, the authors concluded that GSTs are involved in the conjugation of isothiocyanates with GSH under the *in vitro* conditions tested (Wadleigh & Yu, 1988b). Since then, it has been commonly believed that isothiocyanates are detoxified by GST activity in generalist lepidopteran larvae. Yet, unequivocal evidence for the involvement of GSTs in this process is still missing for several reasons. First, the conditions used for GST assays are usually chosen to suppress spontaneous conjugation of GSH with the substrates tested. Low pH (pH 6.5 – 6.8) and equimolar concentrations of GSH and isothiocyanate substrates are commonly employed. However, under physiological conditions pH values between pH 7 and 7.5 and an excess of GSH as compared to isothiocyanates can be expected (Kolm *et al.*, 1995; Meyer *et al.*, 1995; Zhang *et al.*, 1995; Wiktelius & Stenberg, 2007).

Second, although insects were experimentally induced with isothiocyanates, CDNB (1-chloro-2,4-dinitrobenzene) instead of isothiocyanate was frequently used as a substrate in the enzyme assays. The induction of GST activity under these conditions might not necessarily mean a higher activity for isothiocyanate conjugation. Furthermore, the use of these experimental conditions for testing GST activity may lead to significant underestimates in the rate of spontaneous GSH-isothiocyanate conjugation occurring *in vivo*. And, while human GSTs have been characterised with isothiocyanates as substrates, nothing is known about the substrate specificities or enzyme kinetics of lepidopteran GSTs with isothiocyanates as substrates. But, investigating these parameters may offer valuable information on the involvement of GSTs in isothiocyanate metabolism in lepidopteran herbivores.

The goal of this study was to assess the actual contribution of GSTs to isothiocyanate metabolism in generalist lepidopteran herbivores and the role of GSH is in this process. Using the generalist lepidopteran herbivore *Spodoptera littoralis* as a model organism, we investigated formation of GSH-isothiocyanate conjugates in the midgut tissue *in vitro*. We compared the assay conditions (substrates, substrate concentrations, buffer, pH, enzyme concentrations) normally used to test GST activity with conditions prevailing in both the gut lumen and gut cells.

3.2 Materials and Methods

3.2.1 Organisms and Chemicals

Caterpillars of *Spodoptera littoralis* Boisduval (Egyptian cotton leafworm; Lepidoptera, Noctuidae,), obtained from Syngenta (Maintal, Germany), were reared on an artificial diet based on white beans (modified after Bergomaz & Boppre, 1986) at 20°C under ambient light conditions.
L-Glutathione (GSH) as well as all isothiocyanates were acquired from Sigma-Aldrich (Munich, Germany), Alfa Aesar (Karlsruhe, Germany), Calbiochem (Merck KG, Darmstadt, Germany) and Enzo Life Sciences (Lörrach, Germany). All buffer components were purchased from Roth (Karlsruhe, Germany) or Sigma Aldrich. Glutathione disulfide (GSSG) was purchased from Serva (Heidelberg, Germany), glutathione reductase (GR) was from Fluka. 1-Chloro-2,4-dinitrobenzene (CDNB) and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and NADPH all were purchased from Fluka/Sigma.

3.2.2 Chemical syntheses

GSH conjugates of isothiocyanates were chemically synthesised with slight modifications after Kassahun et al. (1997). GSH (5 mg) was dissolved in 0.5 ml 50 % (v/v) ethanol (pH 7.5) and mixed with 1 ml of 0.5 % (v/v) isothiocyanate solution in ethanol. The mixture was agitated under nitrogen on a vortex shaker over night. The identity of the reaction products was confirmed by LC-MS based on the *m/z* of fragments observed in MS^2 and MS^3 spectra.

3.2.3 Preparation of purified midgut GSTs

Third instar larvae of *S. littoralis* were fed on leaves of *Arabidopsis thaliana* (ecotype Col-0) or *Brassica nigra* 24 h prior to dissection to induce GST activity. Just before dissection, caterpillars were chilled at 4 °C for at least 30 min and about 1 min at -20 °C, after which their midguts were removed on ice. Gut contents including peritrophic membranes were discarded. Gut epithelia were ground in liquid nitrogen and extracted in 1 ml of storage buffer (50 mM Tris HCl, pH 7.5, 5 mM EDTA, 20 % glycerol). After centrifugation at 3.800 x*g* and 4 °C for 10 min to remove cell debris, the supernatant was centrifuged at 100.000 x*g* for 60 min at 4 °C to remove all insoluble components. The supernatant was applied to a column of GSH-coupled sepharose (Glutathione Sepharose 4B, GE Healthcare Europe, Freiburg, Germany) according to manufacturer's instructions. The flow-through was collected and reapplied to the GSH column, and this step was repeated eight times (10 passages of the protein extract over the column). The GSH column was subsequently washed with 8 ml phosphate buffered saline, pH 7.4 (PBS; 140 mM NaCl, 2.7 mM

KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) before the GSTs were eluted using 5 ml 50 mM Tris HCl, pH 8.0 including 10 mM GSH. The eluate was then passed over a BioRad PD10 column equilibrated with storage buffer to remove free GSH. The columns were washed with 4 ml storage buffer per 3 ml of applied eluate. The resulting GST preparation was concentrated to 100 to 300 µl by ultrafiltration using Pierce iCON^{TM} Concentrator spin columns (20 ml/9 K; Pierce, Rockford, USA) at 7000 x*g* and at 10 °C. At each step of the purification process, an aliquot was taken for protein concentration determination and for CDNB assays.
Protein concentration was determined with the BioRad protein reagent (Biorad, Munich, Germany), using bovine serum albumin as standard. Protein extracts were kept on ice at all times.

3.2.4 Determination of non-enzymatic conjugation

In a final volume of 200 µl, benzyl-ITC (0.1 mM) and GSH (0.01 mM) were mixed and incubated at 25 °C and 300 rpm for 30 min. Reactions were stopped by addition of 20 µl 5 N HCl. Reaction mixtures were analysed on HPLC and relative amounts were compared. In this experiment, a range of buffers were used at 50 mM, covering a pH range from pH 5 to pH 10.

3.2.5 CDNB assays

CDNB assays were performed according to the Sigma GST Assay Kit with slight modifications. A quantity of 2 µg of protein was used with 1 mM CDNB (40 mM stock in ethanol) and 2- 3 mM GSH (100 mM stock in water) as substrates in a total volume of 1 ml 100 mM K_2PO_4, pH 6.5. Addition of GSH started the reaction. After a 60 sec incubation time, absorbance was read continuously at 340 nm for 150 sec. GST activity (enzymatic rate in µmol* min^{-1}µg $protein^{-1}$) was determined in relation to a buffer control sample as ΔA340/min.

3.2.6 Determination of total GSH content

To assess which GSH concentrations should be used in GST enzyme assays, naturally occurring concentrations of glutathione (GSH + its dimer GSSG) were determined in *S. littoralis* midguts. Midgut epithelia (gut wall) and peritrophic membranes containing the food bolus (gut content) of 20 to 25 day old *S. littoralis* caterpillars were analysed individually. Caterpillars were fed on artificial bean-based diet. In two experiments, caterpillars received diet containing 3 µmol*g^{-1} diet of 2-phenylethyl (PE)-ITC to investigate the effects of isothiocyanate on cellular GSH content. Each sample of gut wall, or gut content respectively, was crushed in 400 µl of extraction buffer (0.1 M potassium phosphate, 5 mM EDTA, 0.1 % Tween X-100 and 0.6 % sulfosalicylic acid, pH 7.5) in a Potter homogenizer with 30 strokes. Coarse cell debris was removed by centrifugation for 10 min at 10,000 x *g*. Three different dilutions of the supernatant were used for GSH measurement. Protein concentration was determined as described above.

Total GSH content was determined following the protocol of the Glutathione Assay Kit (Sigma-Aldrich, Munich, Germany) with the following modifications. Assay buffer (0.1 M potassium phosphate, 1 mM EDTA, pH 7.0) was prepared in our hands. A standard dilution series of 0.625 to 40 µM GSH was employed. Samples were prepared in an extraction buffer as described above. To ensure that the measurement worked correctly, two positive control samples containing known concentrations of GSH and GSSG were always run along with the unknown samples. Measurements were performed in 96 well-plates (Corning® 96 well clear flat bottom UV-transparent microplates, Corning, New York, USA) on a Tecan Infinite M200 photometer (Tecan Group Ltd., Männedorf, Switzerland). A quantity of assay buffer (10 µl as solvent blank), standard curve samples, positive control samples and gut samples were incubated with 150 µl working solution (0.1 mM DTNB, 0.115 units/ml glutathione reductase in assay buffer) for 5 min. After addition of 50 µl 0.2 mM NADPH (in assay buffer) absorbance of samples was immediately read at 412 nm every 30 sec for 5 min with five reads per well.

The values from the GSH standard solutions were used to generate a standard curve from which the values of the unknown samples were calculated based on ΔA412/min. Results are given as µmol GSH *mg protein^{-1}.

3.2.7 GST enzyme assays with isothiocyanates as substrates

Assays were conducted using GST preparations (2 µg protein) with GSH and different isothiocyanates as substrates in a total volume of 200 µl of MES, bis-tris propane and CAPSO buffer at 50 mM. Butyl-, PE-, benzyl- and 4msob-ITC were used as substrates, dissolved in DMSO (4msob-ITC) or ethanol (other isothiocyanates); organic solvent content never exceeded 2 % of the total volume. Assays were incubated at 25 °C and 300 rpm in an incubation shaker. The reaction was stopped by addition of 20 µl 5 M HCl. After centrifugation at 14,000 x *g* for 10 min, 50 µl of the supernatant were analysed by HPLC.

3.2.8 Testing influence of different isothiocyanates on rate of conjugation

Assays were performed as described above at pH 6, 7.4 and 10 using 50 mM buffers (MES, bis-tris propane and CAPSO respectively). GSH and isothiocyanates were used as substrates at concentrations of either 0.01 or 2 mM. The low concentration of one substrate was paired with the high concentration of the other substrate. Isothiocyanates used were 4msob-, benzyl- or PE-ITC.

Reaction rates were determined by HPLC analysis of the conjugates formed, quantified against an internal conjugate standard. Comparison of the reactivity of isothiocyanates was accomplished by subtracting non-enzymatic reaction rates from those catalyzed by GST.

3.2.9 Quantification of conjugate formation in enzyme assays by HPLC

All HPLC analyses were carried out on Agilent 1100 series equipment (Agilent Technologies, Böblingen, Germany). Separation was accomplished using a EC 250/4.6 Nucleodur Sphinx RP column (25 cm x 4.6 mm, 5 µm; Macherey-Nagel, Düren, Germany) with a gradient of 0.2 % (v/v) formic acid (solvent A) and methanol (solvent B). Separation was accomplished at a flow rate of 1.0 ml*min^{-1} at 25 °C as follows: 15- 99 % B (14 min), 99- 100 % B (0.1 min), a 2 min hold at 100 % B and a 4 min hold at 15 % B. The eluate was monitored by diode array detection at 254 nm. Conjugates were identified by retention time in comparison to known standards (see *Chemical syntheses*). Quantification was done relative to an internal standard (a GSH conjugate of an isothiocyanate not used as substrate in the assay) based on peak area at 254 nm, assuming equal response factors.

3.2.10 1- and 2-D SDS PAGE gels of GSTs

After purification as described above, GSTs (for amounts see Fig. 2) were mixed with 3x SDS loading buffer, boiled for 10 min, cooled on ice and the resulting precipitate centrifuged at 14,000 x*g* before being loaded on to pre-cast gradient SDS PAGE gels (4- 15 % acrylamide; BioRad Ready Gels, BioRad, Hercules, USA). After electrophoresis, gels were fixed in 40 % ethanol, 10 % acetic acid for at least 30 min, and subsequently stained with Coomassie colloidal stain (prepared according to the GE Healthcare 2-D Electrophoresis manual, Freiburg, Germany).

For 2-D gel electrophoresis, purified GSTs (see *Preparation of purified midgut GSTs*) were treated with the 2-D Clean-Up Kit (GE Healthcare) according to protocol A and finally suspended in 250 µl rehydration buffer (7 M urea, 2 M thiourea, 2 % CHAPS, 40 mM DTT). After protein concentration determination (2-D Quant Kit; GE Healthcare), 250 µl GST preparation (50 µg) were loaded on to Immobiline DryStrip gels with a pH interval of pI 4 to pI 7. Reswelling occurred over night.

Isoelectric focusing (IEF) was done on an Ettan IPGphor III (GE Healthcare) following the preset program for IEF-strips on this machine. The second dimension was run on hand-cast SDS PAGE gels (15 % acrylamide) with a size of 16 cm x 18 cm x 0.75 mm. After electrophoresis, IEF-strips were removed and SDS gels were fixed and stained with Coomassie stain as described above. Clearly defined protein spots were picked and amino acid sequences analysed via MALDI TOF-MS (matrix-assisted laser desorption ionisation-time of flight-mass spectrometry) after trypsin digestion. Processing of protein spots and analysis on MALDI TOF-MS was done according to Pauchet *et al.* (2008).

3.3 Results

3.3.1 An extract enriched in glutathione-S-transferases (GSTs) was prepared from *Spodoptera littoralis* midguts

To study GSTs involved in the metabolism of ingested isothiocyanates, soluble protein was extracted from the midgut after midgut content including peritrophic membranes were removed. Partial purification of GSTs from this extract was achieved by passage over glutathione (GSH)- coupled sepharose columns. The eluate produced a band of 25 kDa in SDS PAGE gels (Fig. 2A). Separation of this eluate in two dimensions showed a reproducible pattern of protein spots at about 25 kDa (Fig. 2B).

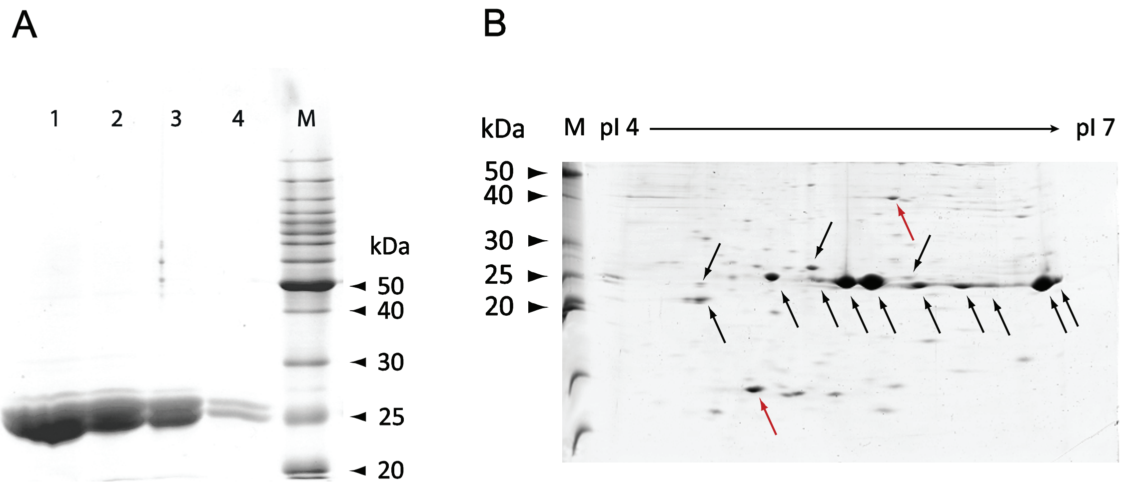

Fig. 2: Analysis of a partially-purified preparation of GSTs from *Spodoptera littoralis* midguts that had been chromatographed over a GSH-sepharose column.

A) 1-D SDS PAGE gel. Samples were loaded at 12, 7.2, 3.6 and 1.2 µg on lanes 1 to 4. Lane M contained 3 µl of marker proteins.

B) 2-D SDS PAGE gel. 50 µg GSTs were loaded and separated on a 13 cm strip from pI 4 to pI 7 during IEF. Lane M contained 5 µl of marker proteins. Spots of 25 kDa identified as GSTs by MALDI-TOF mass spectrometry are marked by black arrows; those with different sizes are marked by red arrows.

In a BLAST-search against public and in-house protein databases, 13 of these protein spots were identified as GSTs after trypsin-digestion and analysis with MALDI-TOF-MS (marked with black arrows in Fig. 2B). Two other spots with higher and lower kDa (red arrows in Fig. 2B) were also identified as GSTs.

The other spots visible could not be identified. Aliquots from raw extracts and the partially purified GST fractions (2 µg respectively) were assayed using GSH and the widely used synthetic substrate, CDNB. These assays demonstrate that purification factors of 2.7 to 40 fold were achieved (Table 1).

Table 1: Activity of partially purified GSTs from *S. littoralis* midguts. Specific activity of 2µg protein aliquots from each step was measured spectrophotometrically at 340 nm, using 3 mM GSH and 1 mM CDNB as substrates. Results are presented as reaction rates in µmol*min^{-1}µg protein^{-1} for four representative purifications.

experiment	raw extract	GSH-sepharose column-flow-through	GSH-sepharose column-eluate	purification factor
A	1.35	0.96	17.62	12.6x
B	0.82	0.32	32.80	40x
C	0.85	0.11	30.43	35.8x
D	0.36	0.23	12.4	31x

3.3.2 GST-driven conjugation with isothiocyanates depends on both pH and relative substrate concentration

To determine where GST-catalyzed GSH-isothiocyanate conjugate formation might take place in *S. littoralis*, enzyme assays testing an array of different conditions were performed with various isothiocyanates (Fig. 3).

Both substrate ratios and pH were varied to cover a multitude of *in vitro* conditions. Assay results are displayed as the ratio between conjugate formation in the presence vs. the absence of GSTs, i.e. a ratio of enzymatic to non-enzymatic conjugation. A ratio of >1 demonstrates GST activity because enzymatic conjugation exceeds non-enzymatic product formation. A ratio of one means that the rates are the same and that GST activity is not detectable.

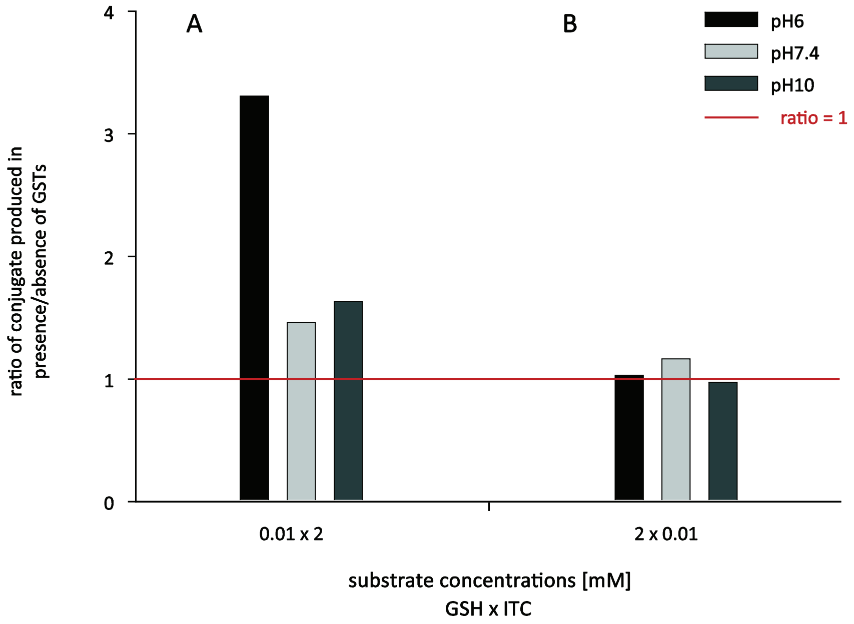

Fig. 3: Enzymatic conjugation between GSH and isothiocyanates varies with pH and substrate concentrations.

Assays were performed in a 200 µl volume with buffers of 50 mM MES (pH 6), CAPSO (pH 10) and bistris propane (pH 7.4). GSH and benzyl-ITC were added in combinations at 0.01 mM (low) or 2 mM (high). All results represent the means of two replicates. Experiments performed with PE-ITC and 4msob-ITC gave very similar results.
Results are presented as the ratio of activity in the presence of GST-enriched extract vs. the absence of extract. A ratio of 1 (red line) and below means there is no detectable GST activity, a ratio above 1 indicates GST activity.

GST activity was only detectable when isothiocyanate concentration was high and GSH concentration was low (Fig. 3, left side of graph). Under these conditions, reactions at pH 7.4 and pH 10 yielded some detectable GST activity, whereas at pH 6 enzymatic activity was higher. When isothiocyanate concentration was low and GSH concentration high, GST activity was undetectable regardless of pH (right side of graph). Thus, a low pH with high isothiocyanate and low GSH concentrations made the contribution of GSTs to the conjugation of the substrates most visible *in vitro*.
To determine more precisely the substrate concentrations giving maximal GSH-isothiocyanate conjugate formation under GST catalysis, assays were run at pH 6 using a variety of GSH to isothiocyanate ratios (Fig. 4). GST activity was detectable at all chosen substrate ratios with the highest activities at the lowest GSH concentrations.

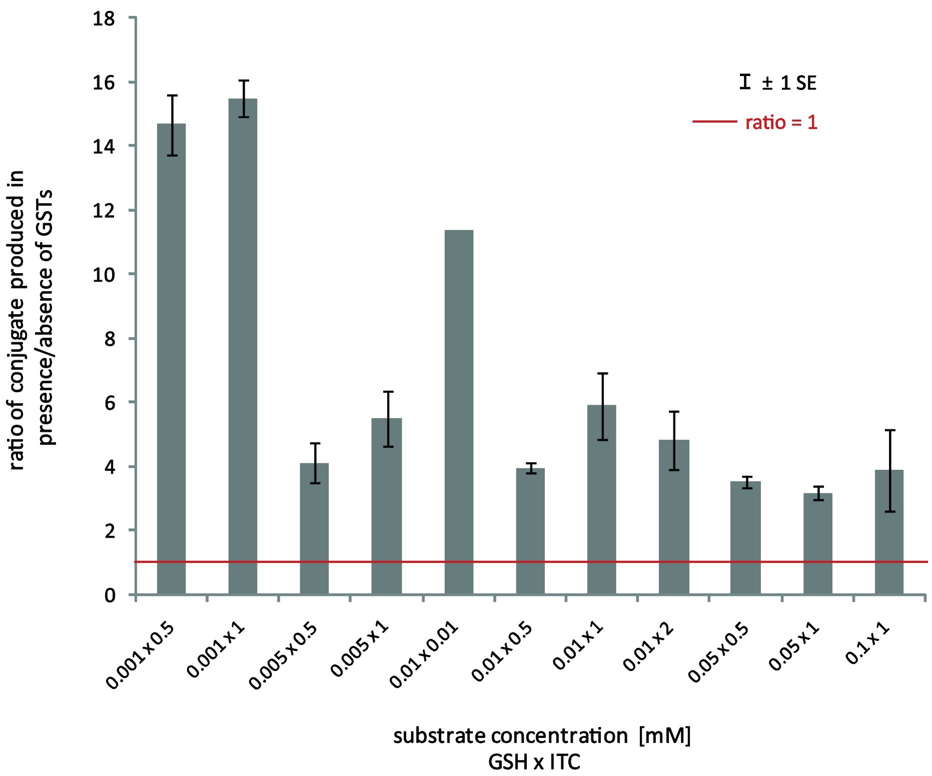

Fig. 4: Effect of changing GSH : isothiocyanate ratios on GST activity.

Assays were performed in a 200 µl volume of MES buffer (50 mM), pH 6. GSH and benzyl-ITC were added in combinations as shown. Results are presented as the ratio of activity in the presence of GST-enriched extract vs. the absence of extract. A ratio of 1 (red line) or below means there is no measurable GST activity, a ratio above 1 indicates GST activity.
The graph combines results (means ± 1 SE) for several different enzyme preparations, each of which was assayed in duplicate. Three different isothiocyanates were tested, benzyl-, PE-, and 4msob-ITC, with similar results which are combined here. In some cases, no conjugate was detectable in the absence of enzyme so no ratio of enzymatic to non-enzymatic conjugation could be calculated (this would require division by zero). These replicates are not included here.

3.3.3 Isothiocyanate side chain structure influences conjugate formation by GSTs

Isothiocyanates have very different chemical structures depending on the glucosinolate they are derived from. Since these structural differences might influence their suitability as substrates for GSTs, we compared three different isothiocyanates (benzyl-, PE- and 4msob-ITC) as substrates for GSTs at pH 6 at two different substrate concentrations. The results are displayed as the ratio of conjugate formation in the presence of GST-enriched extract over absence of extract (Fig. 5).
As shown, when pH was varied, GST activity was consistently higher at low (0.01 mM) GSH and high (2 mM) isothiocyanate concentration than when the substrate proportions were reversed. PE-ITC exhibited the greatest conversion of all isothiocyanates for both substrate proportions followed by benzyl- and 4msob-ITC at low GSH concentration.

When GSH concentration was high (Fig. 5B), benzyl- and 4msob-ITC both yielded similarly low activities. The specific reaction rates for each isothiocyanate at pH 6, presented in Table 2, reflected the results displayed in Fig. 5, although differences between isothiocyanates are more apparent.

Table 2: Reaction velocity (µmol*$\mu g^{-1}min^{-1}$) of *S. littoralis* midgut GST preparation with various isothiocyanates at different isothiocyanate to GSH ratios and different pHs. Enzymatic reaction rates were corrected for non-enzymatic conjugation. The highest specific activity for each combination is marked in red. ND: GST activity not detectable under these assay conditions.

	isothiocyanate	pH 6 (MES)	pH 7.4 (bis-tris propane)	pH 10 (CAPSO)
2 mM ITC x 0.01 mM GSH	**benzyl-ITC**	0.0013	0.0156	ND
	PE-ITC	0.0492	0.006	ND
	4msob-ITC	0.0069	0.0117	0.006
0.01 mM ITC x 2 mM GSH	**benzyl-ITC**	0.0726	0.0553	0.0353
	PE-ITC	0.0827	0.0543	0.0504
	4msob-ITC	0.0203	0.0496	0.0304

3.3.4 Non-enzymatic conjugation depends both on pH and relative reactant concentration

From the data presented above, it can be concluded that GST activity towards isothiocyanates is best detected at low pH, low GSH and high ITC concentrations. But under other conditions, non-enzymatic reaction rates are higher than enzymatic ones (Fig. 5B). To investigate in more detail which *in vitro* conditions favour non-enzymatic conjugation of GSH and isothiocyanates, we varied pH and relative reactant concentrations.

In a first experiment, pH values ranging from pH 5 to pH 10 were tested with a variety of buffers. The nature of buffers did not influence spontaneous conjugation (Fig. 6), yet pH had considerable impact on the amount of product.

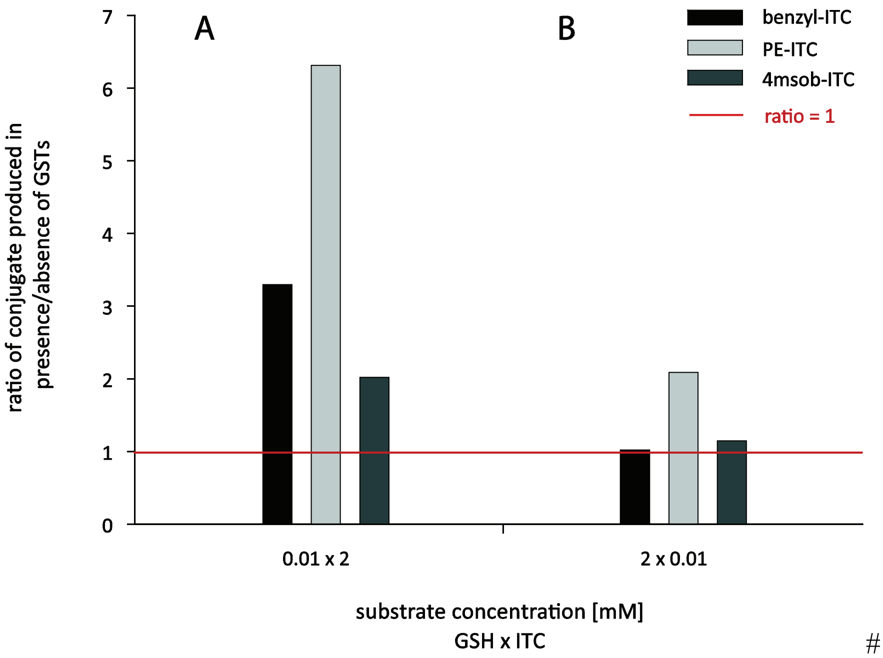

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Fig. 5: Influence of isothiocyanate side chain structure on GST-mediated conjugate formation.

Benzyl-, PE- and 4msob- isothiocyanate were tested as substrates for *S. littoralis* midgut GSTs at pH 6.0 under two ratios of GSH to isothiocyanate substrates. GSH and isothiocyanates were used at 0.01 mM (low) and 2 mM (high) and combined as displayed. Results are presented as the ratio of activity in the presence of GST-enriched extract vs. the absence of extract. A ratio of 1 (red line) or below means there is no measurable GST activity, a ratio above 1 indicates GST activity.

While at slightly acidic to neutral pH (pH 5.5 to 7.0) only little non-enzymatic product was detectable, conjugation steeply increased towards pH 7.0 and higher (Fig. 6). Between pH 9 and pH 10, spontaneous conjugation slightly decreased again. Therefore, at physiological pH values such as pH 7.4 (intracellular pH) or pH 10 (pH in the gut lumen of *S. littoralis*), a high non-enzymatic background to GST-driven reactions should be expected.

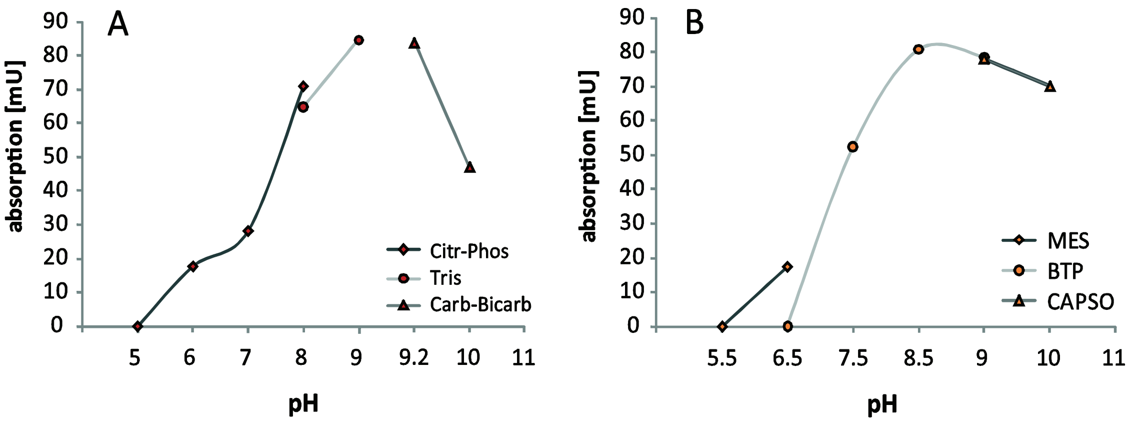


Fig. 6: Non-enzymatic GSH-isothiocyanate conjugation rate varies with pH.

The spontaneous (non-enzymatic) conjugation rate of GSH and benzyl-ITC was assessed in two series of biological buffers of 50 mM concentration. Each assay contained 0.01 mM GSH and 0.1 mM isothiocyanate in a total volume of 200 µl. Results represent the means of three replicates and are displayed as absolute units of absorbance. Citr-Phos: citrate-phosphte; Carb-Bicarb: carbonate-bicarbonate; MES: 2-(N-morpholino)-ethanesulfonic acid; BTP: bistris propane; CAPSO: 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid.

In a more detailed experiment, both pH and reactant concentration were varied to determine their effect on spontaneous GSH-isothiocyanate conjugation. We employed benzyl isothiocyanate and GSH at concentrations of either 0.01 mM (low) or 2 mM (high) in a series of low x low (Fig. 7A), low x high (Fig. 7B), high x low (Fig. 7C) and high x high (Fig. 7D). These concentrations include conditions where high GST activity was detected. pH conditions were chosen to include those of highest GST activity (pH 6), and those simulating intracellular pH (pH 7.4) or gut lumen pH (pH 10). In general, low conjugation rates were found with the low x low combination, intermediate rates with the low x high and high x low combinations, and the highest rates with the high x high combination.

The effects of pH showed in all reactant combinations, although they were more pronounced when GSH was low. When GSH was low, non-enzymatic conjugation rates were highest at pH 7.4, less at pH 10 and lowest at pH 6.0. When GSH was high, as would be expected intracellularly, non-enzymatic rates were highest at pH 10, and slightly lower at pH 7.4 and pH 6.0, successively.

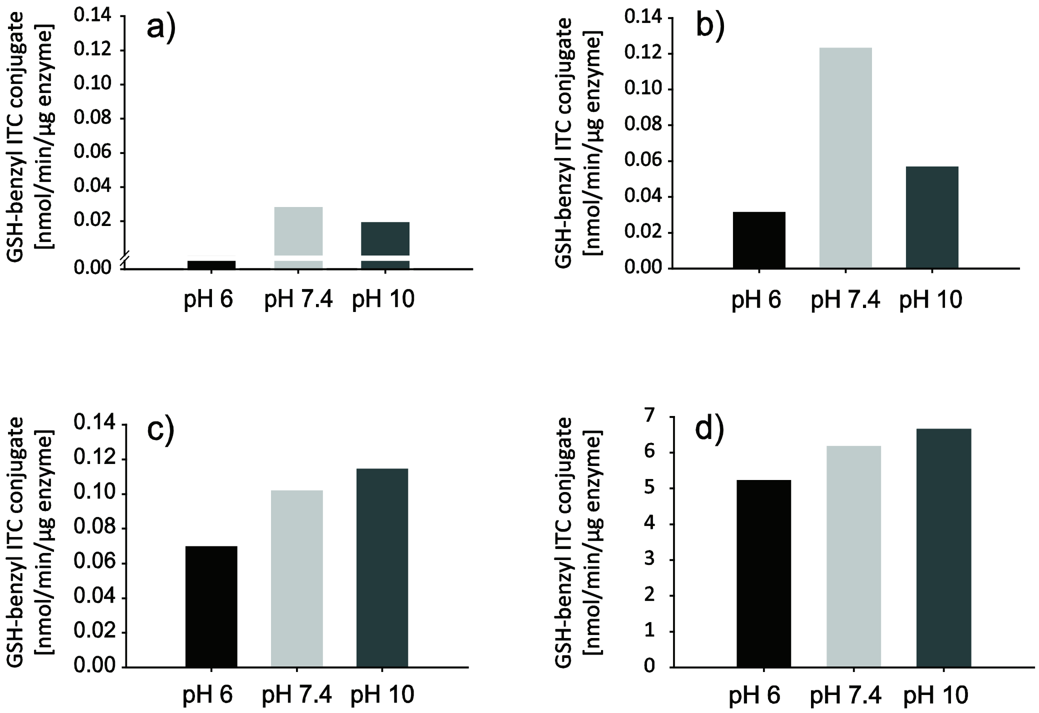


Fig. 7: Non-enzymatic GSH-isothiocyanate conjugation depends on both pH and reactant concentrations. Assays were performed with buffers (50 mM) at pH 6 (MES), pH 10 (CAPSO) and pH 7.4 (bistrispropane). Reactants were added in combinations of 0.01 mM benzyl-ITC and 0.01 mM GSH **(a)**, 2 mM benzyl-ITC and 0.01 mM GSH **(b)**, 0.01 mM benzyl-ITC and 2 mM GSH **(c)**, and 2 mM benzyl-ITC and 2 mM GSH **(d)**. Results represent the means of two replicates. Similar results were obtained for PE- and 4msob-ITC. Note different scale in part d.

3.3.5 Under physiological conditions GST activity cannot be shown

The reaction of isothiocyanates and GSH, either GST-catalyzed or non-enzymatic, could take place in two principal locations in insects: within cells, characterised by pH 7.4, a 2-10 mM concentration of GSH (Josephy & Mannervik, 2006) and a relatively low concentration of isothiocyanates (Zhang, 2000; Zhang, 2001), and in the gut lumen before uptake into cells, characterized by pH 10 in *S. littoralis* and other insects, and GSH and isothiocyanate concentrations which mostly depend on the larval diet. We carried out more detailed experiments under these conditions to determine the relative extent of enzymatic *vs.* non-enzymatic conjugation.

For intracellular conditions as defined above, assays were performed at pH 7.4 using different substrate ratios, chosen to combine a realistic GSH concentration (2 mM) with an equal or lower isothiocyanate concentration. Regardless of substrate concentrations, GST activity was not detectable under these conditions (Fig. 8).

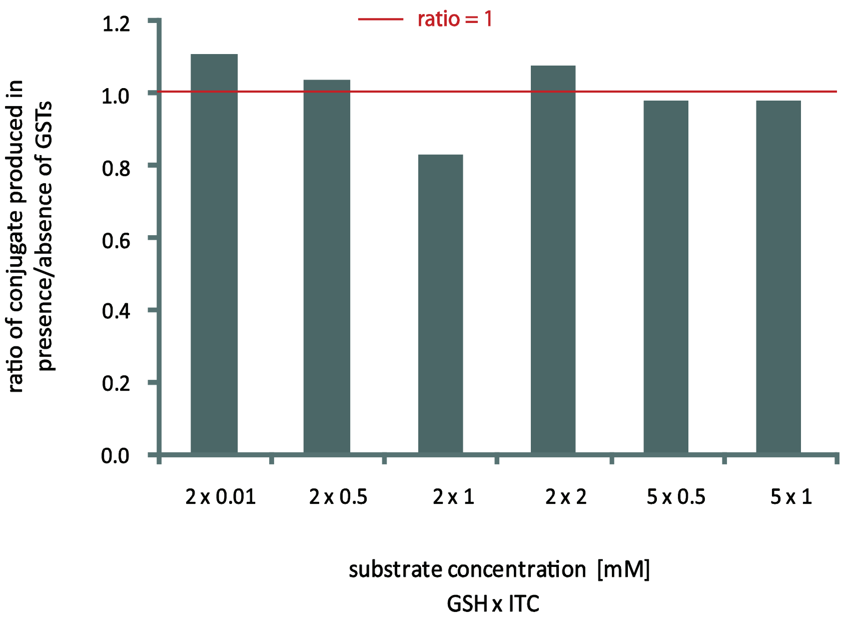


Fig. 8: GST activity is not detectable under intracellular conditions.

Assays were performed in bistris propane buffer (50 mM), pH 7.4 with GSH and isothiocyanates added in combinations as shown.

Results are presented as the ratio of activity in the presence of GSTs to that in the absence of GSTs. A ratio of 1 (red line) or below means there is no measurable GST activity; a ratio above 1 indicates GST activity. The graph combines results for several different enzyme preparations, each of which was assayed in duplicate. Three different isothiocyanates were tested, benzyl-, PE-, and 4msob-ITC, with similar results which are combined here.

For the gut lumen conditions as described above, four substrate combinations were tested using three different isothiocyanates (benzyl-, PE- and 4msob-ITC), all at pH 10. Under these conditions, GST activity was generally not detectable when GSH concentration was high (2 mM) and detectable at moderate levels relative to non-enzymatic conjugation when GSH concentration was low (0.01 mM) (Fig. 9). Activity was highest with PE-ITC.

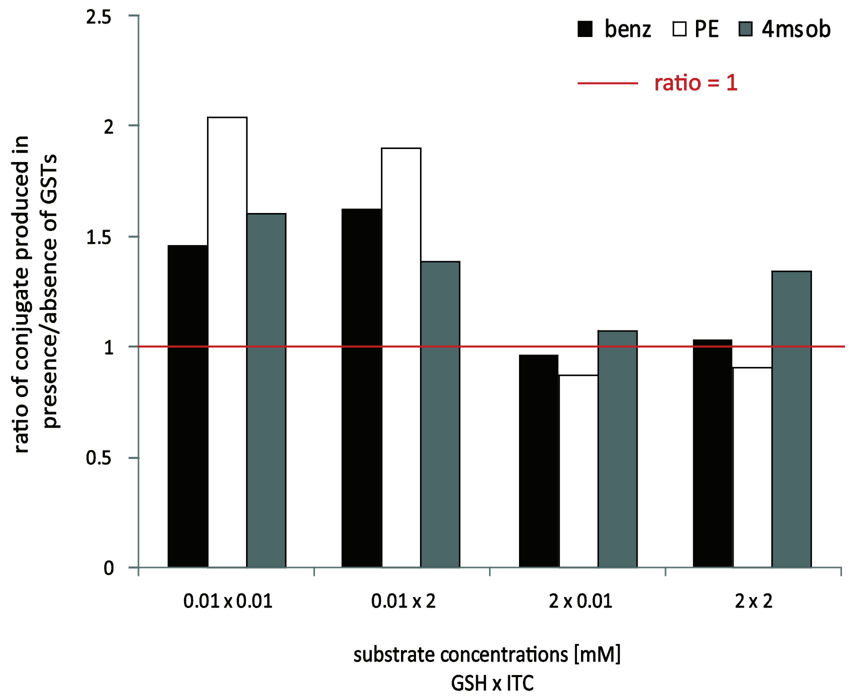


Fig. 9: GST enzyme assays at gut lumen pH.

Assays were performed in a final volume of 200µl CAPSO buffer (50 mM), pH10. GSH and ITCs were added in combinations as displayed. Isothiocyanates used were benzyl-, PE- and 4msob-ITC. Results from all experiments are given as means of two replicates.
Results are presented as the ratio of assays in the presence of GSTs to that in the *absence* of GSTs. A ratio of 1 (red line) or below means there is no measurable GST activity; a ratio above 1 indicates GST activity.

The actual velocities of our *S. littoralis* midgut GST preparation with different isothiocyanates at pH values simulating intracellular and gut lumen conditions, given in Table 2, reflect the patterns in Figs. 8 and 9. At pH 7.4, GSTs have the highest activity using a physiological substrate ratio (with 2 mM concentrations of GSH), but there are no major differences among isothiocyanate substrates. This ratio yielded higher specific activities overall than the ratio using lower GSH (0.01 mM) and 2 mM isothiocyanate. At pH 10, actual velocities were detectable for substrate ratios of 2 mM GSH and 0.01 mM isothiocyanate. PE-ITC had the highest activity, while benzyl- and 4msob-ITC showed similar lower activity.

3.4 Discussion

In specialised insect herbivores, several adaptations to the glucosinolate myrosinase system have evolved which mainly aim at avoiding the generation of isothiocyanates during the feeding process (Ratzka *et al.*, 2002; Wittstock *et al.*, 2004; Muller & Wittstock, 2005). However, larvae of the generalist-feeding lepidopteran noctuid *Spodoptera littoralis* do not possess such specialised proteins. They instead have been shown to use a route of metabolism very similar to that in humans and other mammals (Fig. 1): In faeces of *S. littoralis* and several other generalist lepidopteran herbivores, GSH conjugates of the isothiocyanates they had ingested were found (see Chapter I of this thesis). Several years ago, Wadleigh & Yu (1988b) showed that isothiocyanates lead to a depletion of GSH in enzyme assays with purified GSTs from generalist caterpillars, indicating a participation of those enzymes in isothiocyanate metabolism. In this study, we investigated the contribution of *S. littoralis* GSTs to the conjugation of isothiocyanates with GSH.

3.4.1 Characteristics of GSTs partially purified from Spodoptera littoralis midgut tissue

Partial purification of GSTs from *S. littoralis* midguts usually resulted in a 30-40-fold enrichment based on assays with CDNB (Table 1), a model substrate for GSTs (Ranson & Hemingway, 2005). Purification factors were comparable to those obtained in other studies (Yu, 1989; Lagadic *et al.*, 1993; Francis *et al.*, 2001; Francis *et al.*, 2002).

SDS PAGE gels separating the purified GST-fraction in one (Fig. 2A) and two dimensions (Fig. 2B) show that most proteins in the extracts were approximately 25 kDa, which corresponds to the size of most insect GSTs (Enayati *et al.*, 2005). Reproducible protein spots from 2D protein gels were analysed by MALDI-TOF-MS and 15 of them were identified as GSTs (Fig. 2B). The pattern of spots at 25 kDa largely overlaps with the pattern published by Dabrowska *et al.* (2009) where the authors also identified all spots as GSTs. Since post-translational modifications are rare in GSTs, it can be expected that the spots represent individual GST enzymes (Hayes *et al.*, 2005). In our 2-D gels, several protein spots could not be identified, mostly because not enough protein material could be extracted.

3.4.2 The best conditions to detect GST activity *in vitro* are pH 6 and low glutathione (GSH) concentration

GST activity with isothiocyanates was sought under a range of different conditions, including conditions that might be encountered in the gut epithelium cells (pH 7.4), in the gut lumen (pH 10), and conditions which are commonly used in the biochemical characterisation of GSTs (pH 6).

The best enzyme activity after non-enzymatic conjugation was subtracted was detected at pH 6 with GSH concentrations of 1 or 100 µM (Fig. 3). The concentration of isothiocyanate was not as critical. These results agree with those from several other studies on GST activity with isothiocyanates (Kolm *et al.*, 1995; Meyer *et al.*, 1995; Zhang *et al.*, 1995; Zhang, 2001; Nutricati *et al.*, 2006; Wiktelius & Stenberg, 2007). Concentrations of GSH greater than 100 µM may lead to substantial non-enzymatic conjugation, especially when isothiocyanate concentration is low, thus obscuring enzyme catalysis and conversion.

At pH 6, the order of preference of GSTs for isothiocyanates is 2-phenylethyl (PE)-ITC > benzyl-ITC > 4msob-ITC (Fig. 5A; Table 2). This preference may be explained by substrate size, aromaticity or polarity or any combination of those factors. Interestingly, isothiocyanate toxicity has been shown to increase with molecular size (Wadleigh & Yu, 1988b) and aromaticity (Borek *et al.*, 1998). Thus, of the substrates tested, PE-ITC should be the most toxic; this was also the most preferred substrate. Isothiocyanate structure affected enzyme conversion rates only when GSH concentration was low (Fig. 5A), perhaps because this suppressed non-enzymatic conjugation which might mask differences between isothiocyanates. The fact that GST activity was detected only at conditions entirely different from what is expected in natural systems (Fig. 3) indicated that GSTs may not play as big of a role in isothiocyanate conjugation *in vivo* as previously believed.

3.4.3 The rate of non-enzymatic conjugation of GSH with isothiocyanates is higher than that catalyzed by GSTs at physiological conditions

As described above, GST activity was also assessed under assay conditions simulating the physiological environment of the gut lumen and the midgut epithelial cells since these are the locations where isothiocyanates are most likely to encounter GSTs. Intracellular conditions are characterised by pH 7.4 while the pH in the lumen of *S. littoralis* midguts ranges around pH 10 (L. Ping, pers. communication). At these pH values, low or no net enzyme activity measured (Fig. 3). It has been previously noted that spontaneous reaction of GSH with isothiocyanates occurs at a high rate at basic pH (Wadleigh & Yu, 1988b; Meyer *et al.*, 1995).

Hence, the rate of non-enzymatic conjugation was studied in more detail for a range of pH values. These data showed that spontaneous conjugation is highest between pH 8 and pH 9 (Fig. 6). Yet at pH 7.4 and pH 10 non-enzymatically formed conjugate still is made at a substantial rate compared to pH 6. Any enzyme assay performed at a pH > 7 will experience a large non-enzymatic background. And from these data, it appears very likely that isothiocyanates are subject to non-enzymatic conjugation with GSH, both within cells and in the gut lumen.

In addition to pH, we also investigated the GSH concentrations to be expected intracellularly and in the gut lumen. In human cells, GSH concentrations between 2 and 10 mM are common. Measurements of GSH (GSH + GSSG) in *S. littoralis* gut tissue revealed a high concentration: 30 nmol*µg protein^{-1} (see Chapter III), a value that seems to be higher than that measured in other insect herbivores (Barbehenn, 2003; Jovanovic-Galovic *et al.*, 2004). In contrast, the concentration of isothiocyanates in cells should be lower than that of GSH because isothiocyanates accumulate within cells predominantly as GSH conjugates and not as free isothiocyanates (Zhang, 2000; Zhang, 2001). In the midgut, isothiocyanate concentration will vary depending on the plant material ingested and the efficiency of glucosinolate hydrolysis. As GSH transport out of cells is normally very limited (Griffith, 1999), GSH concentrations in the lumen should be low. In fact, measurements of GSH concentration in the gut contents revealed that there was no detectable GSH present (data not shown; detection limit: 0.625 µM were reliably measured). Yet, GSH might be present in the food ingested, especially in plant material. Therefore depending on the larval diet, some quantity of GSH could be present in the food bolus.

The effects of pH and reactant concentration on non-enzymatic GSH-isothiocyanate conjugation were tested at various pH values with a grid of GSH and isothiocyanate concentrations representing physiological and non-physiological conditions. The results confirmed preliminary findings, showing that non-enzymatic conjugation was strongly dependent on pH and GSH concentration (Fig. 7). These results show vividly how much influence pH and reactant concentrations have on the outcome of the reaction *in vitro* and illustrate how difficult it may be to convincingly demonstrate GST activity for GSH-isothiocyanate conjugation under physiological conditions using *in vitro* assays.

When GSTs were tested under physiological conditions, GST participation in GSH-isothiocyanate conjugation was virtually not detectable (Figs. 8 and 9), especially with the high GSH concentrations (2 mM) used in *in vitro* assays. As GSH concentrations are in this range in most living cells, including insect cells, this observation suggests that GSTs may play a much smaller role than previously thought in isothiocyanate metabolism.

Since all GST preparations used in our experiments exhibited GST activity with CDNB, we conclude that the enzymatic conjugation with isothiocyanates was masked by a high non-enzymatic background in assays simulating physiological conditions. Of the published studies using isothiocyanates as substrates for GSTs (Kolm *et al.*, 1995; Meyer *et al.*, 1995; Zhang *et al.*, 1995; Zhang, 2001; Nutricati *et al.*, 2006; Wiktelius & Stenberg, 2007), none evaluated GSTs under different pH regimes, although Kolm *et al.* (1995) mention that physiological pH tends to be higher than the pH at which they usually conducted their assays (pH 7.5 compared to pH 6.5). These authors report that while non-enzymatic background is higher at pH 7.5 than at pH 6.5, GST activity is also higher so that GSTs contribute as much to the conjugation at physiological pH as at pH 6.5. However, they do not show any data to support this assertion. Based on our results, it seems that their conclusion is not applicable to the *S. littoralis* GST preparations tested here.

When the conjugation rate of structurally different isothiocyanates was tested, no differences were found (Fig. 8). This was also reflected in the specific activities of the GST preparation for different isothiocyanates, especially when GSH concentration was chosen to be within the physiological range (Table 2). Thus it can be concluded that under physiological conditions all isothiocyanates are conjugated with equal efficiency.

The results from this section reveal clearly why most GST activity assays so far have been conducted at a slightly acidic pH. The low pH makes it possible to detect GST activity at all. Yet, to find out if GSTs play a role in the first step of isothiocyanate metabolism in insect herbivores, *in vitro* assays should simulate *in vivo* conditions as closely as possible. This means using physiological pH values and substrate ratios which reflect natural conditions. While GSTs were present and active in gut tissue, GST activity in the gut lumen appears hardly probable as insect GSTs are mostly cytosolic enzymes (Enayati *et al.*, 2005). In agreement with this, most GST sequences from *S. littoralis* midguts apparently lack localisation signal peptides (pers. comm. Heiko Vogel) which would direct the enzymes away from the cytosol. Thus, it is unlikely to find GSTs outside of cells in the gut lumen. In the absence of GSTs, conjugation of isothiocyanates with GSH in the gut would likely occur in a non-enzymatic way. Yet, even in the presence of GSTs in gut epithelial cells, non-enzymatic conjugation is nonetheless the more likely route of isothiocyanate metabolism, due to the favourable conditions as illustrated by our results.

Nevertheless, the fact that GST activity above non-enzymatic background could not be demonstrated under the chosen *in vitro* conditions could be due to the absence of some factors present only *in vivo*. Ketterer (1982) argued that the very high GST concentrations in natural systems can make up for the low catalytic properties of this enzyme family. Experiments comparing the effect of increasing GST concentrations, while leaving other conditions constant, could shed light on this for *S. littoralis*.

Furthermore, the mixture of partially-purified GSTs used here could mask effects which become visible only when individual GSTs are biochemically examined. Individual GST enzymes might interact or inhibit each other in a mix and thus produce artefacts absent in a single-enzyme preparation. Since many GSTs have functions not related to xenobiotic metabolism (Frova, 2006; Dourado *et al.*, 2008; Allocati *et al.*, 2009; Dabrowska *et al.*, 2009; Morel *et al.*, 2009), some of the GSTs from *S. littoralis* midgut tissue (Fig. 2B) may not accept isothiocyanates as substrates and thus distort our perception of the properties of the mixture. In future studies, *S. littoralis* midgut GSTs should be re-assessed individually using heterologous expression of cloned genes.

3.4.4 A model of isothiocyanate conjugation in generalist insect herbivores

The involvement of GSTs in isothiocyanate metabolism in generalist caterpillars has been widely accepted since the publications of Wadleigh & Yu (1988b) and Yu (1982; 1983). However, more recent investigations on the metabolism of isothiocyanates in humans (Zhang *et al.*, 1995; Thornalley, 2002; Traka & Mithen, 2009) and the conjugation of isothiocyanates and GSH (Ketterer *et al.*, 1983; Satoh, 1995; Dickinson & Forman, 2002; Lo *et al.*, 2007) have concluded that the process of isothiocyanate conjugation in humans is still not entirely clear. While it is commonly accepted that isothiocyanates produced in human digestive systems are rapidly but passively absorbed into the gut cells, it remains unclear whether conjugation to GSH is catalysed by GSTs, occurs spontaneously, or both (Traka & Mithen, 2009). Some human GSTs investigated did accept isothiocyanates as substrates (Kolm *et al.*, 1995; Meyer *et al.*, 1995; Zhang *et al.*, 1995), and may thus be involved in this process. However, as we have shown here for *S. littoralis*, the activity of human GSTs is also strongly influenced by the *in vitro* conditions used for assays and assays are frequently performed at acidic pH. Thus for human GSTs as well as for *S. littoralis*, isothiocyanate conjugation may proceed very differently at physiological conditions than at those conditions commonly used for an assay. This concern was voiced by Meyer *et al.* (1995), but not pursued further.

The data presented here support the notion that GSTs do not play such a great role in isothiocyanate conjugation as previously believed. Both GSH and isothiocyanates react readily without a catalyst due to the very electrophilic nature of the isothiocyanate function (Ketterer *et al.*, 1983), with reactivity likely also dependent on the variable side chain (Kolm *et al.*, 1995; Meyer *et al.*, 1995; Satoh, 1995; Zhang *et al.*, 1995; Conaway *et al.*, 2002; Lo *et al.*, 2007). From our experiments, GSTs are not likely to have a major effect on conjugation under physiological pH and GSH concentrations.

Yet due to the GSH-depleting properties of isothiocyanates (Zhang, 2000; Zhang *et al.*, 2005), GSTs may have an influence on the process of isothiocyanate detoxification. We thus propose a model for isothiocyanate metabolism in the midguts of generalist lepidopteran herbivores.

Much of the evidence for our model comes from reports about the properties of mammalian GSTs. Certain classes of cytosolic GSTs (α, μ and π) have been shown to have a low K_m for GSH which should be coupled to a high affinity for GSH and low product-release rates (Meyer, 1993). As a consequence, such GSTs tend to be very sensitive to product inhibition such that the products of catalysis often have a K_i lower than the K_m of either GSH or the electrophile (Meyer *et al.*, 1995; Paumi *et al.*, 2001). Similar results have also been shown for insect and bacterial GSTs (Nay *et al.*, 1999; Allocati *et al.*, 2005). Evolutionarily, these GSTs may be said to have given up catalytic efficiency in favour of increased product binding, which may be useful in detoxification. One likely explanation is that such product-binding GSTs interact closely with transporter proteins that remove GSH conjugates from the cells (Nay *et al.*, 1999; Paumi *et al.*, 2001; Allocati *et al.*, 2005). This interaction makes detoxification and removal of metabolites more efficient. Another possible explanation, especially for electrophiles like isothiocyanates, concerns the reversibility of conjugation. At intracellular conditions, GSH conjugates with isothiocyanates are unstable and easily dissociate. By retaining the conjugation products, GSTs may stabilise them and prevent reformation of free isothiocyanate.

For our model we propose that the conjugation process is mainly located inside of midgut epithelial cells (Enayati *et al.*, 2005). When an insect starts feeding on glucosinolate-containing plant material, the GSH concentrations are initially high. But, with the continuing influx of isothiocyanates and resulting conjugation, glutathione stores get depleted and the GSH conjugates are actively transported out of the cells (Zhang, 2000; Zhang *et al.*, 2005). GSTs will participate in the conjugation of isothiocyanates (Fig. 10A), but because of the great velocity of non-enzymatic conjugation at intracellular conditions, spontaneous conjugation will yield a much greater proportion of the resulting conjugates (Fig. 10B). The conversion of GSSG to GSH and the *de novo* synthesis of GSH will counteract glutathione depletion for a while. But eventually, GSH levels will drop, leading to a relative increase in enzymatic conjugation since this process is more efficient compared to non-enzymatic conjugation when GSH concentration is low (as demonstrated in this chapter) (Fig. 10C). The resulting GSH depletion will have two effects: GSTs will start to retain conjugates to a greater degree and the significance of conjugate transport from the cell will increase. However, low enzymatic as well as non-enzymatic conjugation will continue (Fig. 10D), if only because under intracellular conditions, the conjugates are not stable and tend to dissociate, releasing free isothiocyanate. The continuous reprovision of free isothiocyanate in the cell leads to an extreme and long-lasting GSH depletion- as can be seen from the data presented in Chapter III. This massive depletion may lead to damage by oxidative stress and direct harm from the increase in free isothiocyanates (see Chapter III). The long-term effects of isothiocyanate consumption include malnutrition, reduced growth, retarded development and death.

Efforts should also be made to study the behaviour of the individual *S. littoralis* midgut GSTs that most readily accept isothiocyanates as substrates. It may also be helpful to determine the concentration of GSTs in *S. littoralis* midgut epithelia because enzyme concentration can enhance enzyme activity even for enzymes with low catalytic efficiencies such as GSTs. Whenever possible, all of this should be conducted at physiological conditions and with midgut epithelial tissue to ensure that the results are relevant to the metabolism of isothiocyanates in insects feeding on intact glucosinolate-containing plants.

To test some of the predictions of this model, much additional research is needed to measure the levels of GSH, isothiocyanates, GSH-isothiocyanate conjugates and GST-mediated conjugate formation in insects feeding on different diets. Experiments manipulating the GSH concentration could help to separate the contributions of GSH and GSTs to isothiocyanate metabolism *in vivo*.

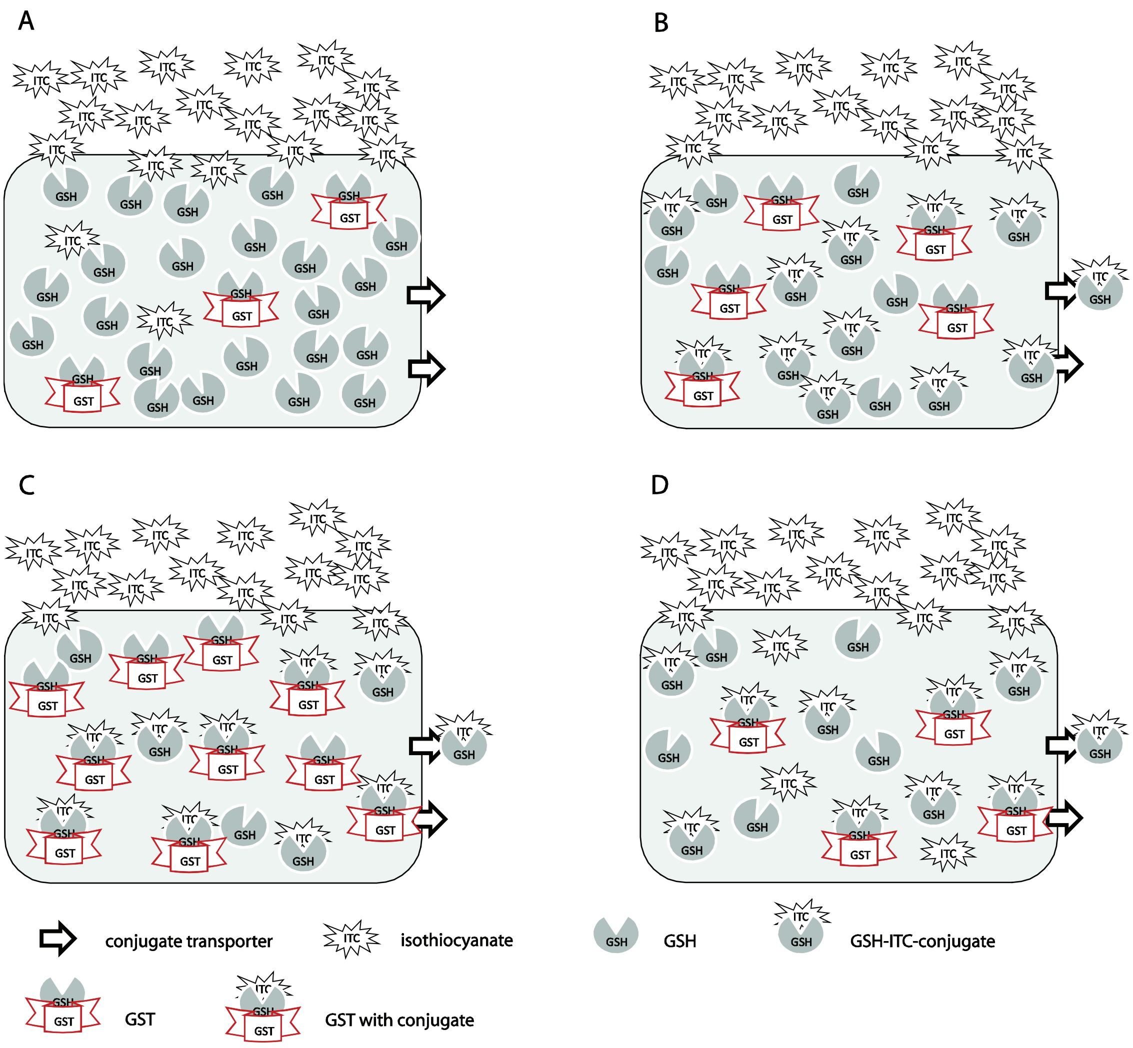


Fig. 10: Proposed model for isothiocyanate conjugation in generalist insect herbivores.

A) When an insect starts feeding on plant tissue containing glucosinolates, GSH concentrations are high. However, a steady influx of isothiocyanates leads to GST-catalyzed isothiocyanate conjugation.

B) Because of the high velocity of non-enzymatic conjugation at intracellular conditions, spontaneous conjugation will constitute the major route for formation of the resulting conjugates. Conversion of GSSG to GSH and the de novo synthesis of GSH provide new glutathione.

C) Eventually, GSH levels drop, leading to an increase in enzymatic conjugation since GST catalysis works more efficiently relative to non-enzymatic conjugation processes when GSH concentration is low. Strong GSH depletion ensues, followed by conjugate retention in GSTs and interaction of GSTs with conjugate transporters removing conjugate from the cell.

D) Low enzymatic as well as non-enzymatic conjugation continues, since under intracellular conditions the conjugates tend to dissociate, releasing free isothiocyanate. The constant supply of free isothiocyanate in the cell leads to an extreme and long-lasting GSH depletion. This massive depletion leads to damage by oxidative stress and direct harm from the free isothiocyanates.

4 Role of glutathione in isothiocyanate processing by the generalist herbivore, *Spodoptera littoralis*

Katharina Schramm, Ute Wittstock and Jonathan Gershenzon

Abstract

Plants of the order Brassicales defend themselves against enemies with an activated plant defense system comprising glucosinolates and myrosinases. The hydrolysis products are known for their biological activity, especially isothiocyanates have been shown to be toxic to a wide range of organisms. Many generalist feeders metabolize isothiocyanates by conjugation with glutathione (GSH). We studied how larvae of the generalist lepidopteran herbivore *Spodoptera littoralis* deal with isothiocyanates in their diet and how detoxification is affected by availability of GSH. When structurally different isothiocyanates were mixed into artificial diets at varying concentrations, larval development (from second larval instar to pupation) was affected in a dose- and structure-dependent manner. Analysis of caterpillar faeces revealed that isothiocyanate metabolism is capacity-limited: high concentrations of 2-phenylethyl isothiocyanate lead to non-linear excretion of free isothiocyanate, accompanied by poor caterpillar performance. Pharmacological manipulation of GSH levels in caterpillar midguts demonstrated that GSH plays an important role for the successful metabolism of isothiocyanates in *S. littoralis* larvae. Further studies need to reveal whether GSH-conjugation of isothiocyanates is the main route of metabolism in generalist insect herbivores and by which mode of action isothiocyanates confer their toxicity.

4.1 Introduction

The glucosinolate-myrosinase system is an activated anti-herbivore defence system of plants from the order Brassicales. In intact plant tissue, the glucosinolate and myrosinase components are separated from each other, but upon tissue damage, they come into contact and the myrosinases hydrolyse the glucosinolates to form an array of biologically active compounds (Fig. 1), amongst them the highly toxic isothiocyanates. The defensive properties of glucosinolate hydrolysis products against insect herbivores are well established (Wittstock *et al.*, 2003; Halkier & Gershenzon, 2006) and isothiocyanates are regarded as one of the most potent groups, exhibiting toxicities comparable to that of synthetic insecticides (Wittstock *et al.*, 2003). Although their *in vivo* mode of action is still unknown, isothiocyanates are known to react with the free amino groups of proteins and to cleave disulfide bonds *in vitro* (Kawakishi & Kaneko, 1987). A number of insect herbivores are specialised feeders on plants of the Brassicales. Investigations suggest that isothiocyanates are as toxic to Brassicales specialists as to other herbivores (Agrawal & Kurashige, 2003), but specialists possess adaptations which minimize exposure to isothiocyanates (Ratzka *et al.*, 2002; Wittstock *et al.*, 2004; Muller & Wittstock, 2005). Some generalist insect herbivores also include Brassicales in their diets, but thus far little is known about how they evade isothiocyanate toxicity.

Humans and rodents can both be considered omnivorous, generalist feeders. In these mammals, isothiocyanates are metabolised to mercapturic acids which are mostly renally excreted. Metabolism is performed by a suite of enzymes known as the mercapturic acid pathway beginning with glutathione-*S*-transferases (GSTs) and ending with *N*-acetyl-transferases, (Zhang, 2000; Conaway *et al.*, 2002; Juge *et al.*, 2007; Traka & Mithen, 2009). In studies with mammalian cell cultures it was shown that isothiocyanates cause a number of intracellular changes. They produce a transient depletion of cellular glutathione (GSH) pools by rapidly reacting with free cellular thiols (Kirlin *et al.*, 1999; Zhang, 2000; Zhang *et al.*, 2005; Odom *et al.*, 2009) thus causing considerable oxidative stress. In addition, isothiocyanates can damage mitochondria (Nakamura *et al.*, 2002; Zhang *et al.*, 2003; Zhang *et al.*, 2005; Xiao *et al.*, 2008) and induce apoptosis (Bonnesen *et al.*, 2001; Clarke *et al.*, 2008; Hayes *et al.*, 2008). After these initial deleterious effects, which might be positive if they occur in cancer cells, isothiocyanates have beneficial consequences, such as the induction of anti-oxidant metabolites, anti-oxidant enzymes and carcinogen-detoxifying enzymes (Zhang *et al.*, 2005; Hayes *et al.*, 2008). GSH plays a significant role in many of these processes due to its high reactivity with isothiocyanates.

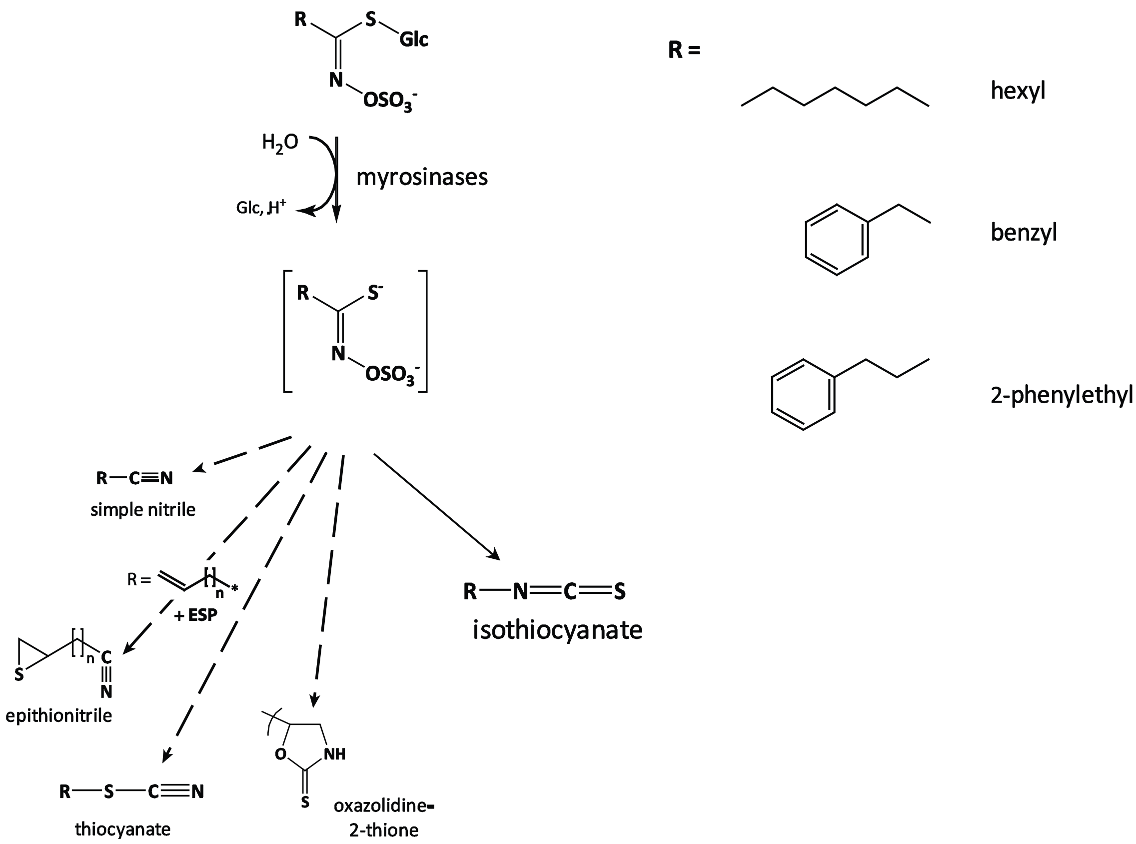


Fig. 1: Glucosinolate hydrolysis.

In intact plant tissue, glucosinolates and their hydrolysing enzymes, the myrosinases, are stored in separate compartments. Upon tissue damage, those two components come into contact forming an array of biologically active compounds. The most extensively studied group is the isothiocyanates. Isothiocyanates (ITC) used in the experiments described here are benzyl-, 2-phenylethyl (PE)- and hexyl-ITC. These isothiocyanates have a low volatility compared to allyl-ITC, which has often been used in previous studies (e.g. Agrawal & Kurashige, 2003). Benzyl- and PE-ITC are derived from aromatic glucosinolates, while hexyl-ITC would be derived from an aliphatic glucosinolate.

Several authors have suggested that isothiocyanates could also be conjugated to GSH in insect herbivores (Yu, 1983; Wadleigh & Yu, 1988; Yu, 1989). In this thesis, GSH conjugates of structurally different isothiocyanates from different plant sources were detected in the faeces of several species of generalist lepidopteran herbivores after feeding on glucosinolates (see Chapter I). Since conjugation of isothiocyanates with glutathione is also the first effect of isothiocyanates described to occur in mammals, the other processes described in mammalian cells (such as induction of anti-oxidants, phase II- detoxification enzymes and enhanced GSH synthesis) could also play an important role in the metabolism of isothiocyanates in insects. Wadleigh and Yu (1988) indirectly demonstrated that the conjugation of isothiocyanates with GSH could be mediated by glutathione-*S*-transferases (GSTs) in lepidopteran herbivores. Yet, in this thesis we have shown that conjugation is likely to be non-enzymatic at least in one herbivore species (see Chapter II). Whether the reaction with isothiocyanates is spontaneous or enzymatic, GSH has a vital role to play in this process, and thus has a critical influence on isothiocyanate toxicity.

Here we investigate the effects of isothiocyanates on the performance of *Spodoptera littoralis* (Boisduval, Lepidoptera: Noctuidae) and what role GSH plays in their detoxification. First, to find out how isothiocyanates affect larval performance, we tested structurally different isothiocyanates fed at the same concentration, and followed caterpillar development from the second larval instar (L2) until eclosion from the pupae. While concentration and structure-dependent toxicity of isolated isothiocyanates on lepidopteran herbivores has been assessed in a previous study (Wadleigh & Yu, 1988), larval development has not been followed over a longer period, an aspect which might be important as many caterpillars complete their development on a single host plant. Second, to assess detoxification capacity towards isothiocyanates, larvae were reared on diet containing different concentrations of a single isothiocyanate, 2-phenylethyl isothiocyanate (PE-ITC). By measuring isothiocyanate-GSH conjugates and metabolites in the faeces in conjunction with larval performance, detoxification capacity was determined.

Finally, to gain a better understanding of the role of GSH in isothiocyanate processing, we pharmacologically manipulated GSH levels in the midgut tissue of caterpillars. *L*-Buthionine sulfoximine (BSO) is a specific and irreversible inhibitor of γ-glutamylcysteine synthase, the enzyme catalysing the final step in GSH biosynthesis (Griffith, 1982; Griffith & Meister, 1985; Griffith, 1999). Treatment with BSO leads to dose-dependent reductions in cellular GSH levels. This effect can last up to 4 days in insects (Fontan *et al.*, 1994; Masuh *et al.*, 1996). Enhancement of GSH levels could conceivably be realized by feeding cysteine as the direct and rate-limiting precursor for GSH biosynthesis. However, since cysteine auto-oxidises to cystine and acts as a cell toxin, it has to be administered in a different chemical form, in human medicine this compound is normally administered as *N*-acetylcysteine (NAC). Caterpillars which had been treated either with BSO or NAC were then challenged with an isothiocyanate-containing diet to follow whether treatments caused a change in detoxification capacity and performance.

4.2 Materials and Methods

4.2.1 Insects & Chemicals

Larvae of *Spodoptera littoralis* (Egyptian Cotton Leafworm; Boisduval), obtained from Syngenta (Maintal, Germany), were reared from eggs on a diet based on white beans (modified after Bergomaz et al) at 21 °C and a light cycle of 12:12 h until used for experiments. All bioassays were performed with *S. littoralis* caterpillars.

Chemicals were purchased from Roth except for hexyl isothiocyanate, reduced glutathione (GSH_{red}), dimerised glutathione (GSSG), NADPH, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), the dipeptide cysteine-glycine (CysGly) and Triton X-100 which came from Sigma-Aldrich (Munich, Germany). *N*-acetylcysteine (NAC), 2-phenylethyl- and benzyl isothiocyanate were bought from Alfa Aesar (Karlsruhe, Germany). *L*-Buthionine sulfoximine (BSO) and sulfosalicylic acid were obtained from Acros Organics (Nidderau, Germany). From Fluka (Buchs, Switzerland), formic acid and GSH reductase (GR) were purchased. All chemicals were of highest available purity.

4.2.2 Glutathione measurements

Total glutathione (GSH = GSH_{red} + GSSG) was measured in *S. littoralis* midgut epithelia. For this, caterpillars were dissected and midguts removed. Midgut epithelia were crushed in a glass potter in 400 µl extraction buffer (0.1 M potassium phosphate buffer, pH 7.5, 5 mM EDTA, 0.1 % Triton X-100, 0.6 % sulfosalicylic acid) with 30 strokes of the pestle. After solids had been pelleted by centrifugation (at 8000 x *g*), the supernatant was diluted with assay buffer (0.1 M potassium phosphate buffer, pH 7.0) and GSH measured following the protocol from Sigma-Aldrich: 10 µl sample or GSH standard was incubated 5 min with 150 µl working solution (assay buffer with 1 mM EDTA, 3.78 mM DTNB and 6 units GR). Then 50 µl NADPH solution (assay buffer with 0.2 mM NADPH) were added to each sample. Immediately, the change in absorbance at 412 nm was measured every 30 sec for 5 min (10 measurements total). From the standards, a curve was calculated and used to quantify GSH in midgut samples. Two positive controls (containing known concentrations of GSH_{red} or GSSG) were run with each measurement to make sure that the procedure worked correctly. Each midgut sample was measured in three dilutions to test for linearity. GSH concentrations were related to total soluble midgut protein to standardise for size differences between caterpillars.

4.2.3 Manipulation of epithelial GSH concentration using BSO and NAC

To test the influence of BSO on the GSH concentration of midgut epithelia, caterpillars were treated with different concentrations of BSO. After 12-18 h of starvation, third instar (L3) caterpillars received 0, 10, 50, 100 or 225 nmol BSO per insect in 2 µl 0.9 % saline dripped onto a small piece of diet. Larvae were allowed to feed on the treated diet overnight. All caterpillars which had consumed at least 80 % of the treated diet were included in the analysis. They received normal diet for 24h and were then dissected and midguts analysed for GSH. This experiment was aimed at finding the concentration of BSO at which the highest GSH depletion would be achieved without caterpillars experiencing toxic effects. We found that the highest concentration was accepted and tolerated well. Therefore, in all other experiments 225 nmol BSO per insect were administered orally (by pipette feeding, Fig. 2).

Fig. 2: Oral administration of *N*-acetylcysteine (NAC) or *L*-buthionine sulfoximine (BSO) treatment to L3 caterpillars.

NAC and BSO were dissolved in a 2 % sucrose solution at 500 mM (NAC) or 125.5 mM (BSO). 2 µl of the solution were fed with a micropipette to unstarved larvae, for a total intake of 1 µmol NAC or 225 nmol BSO per caterpillar. Control larvae received 2 µl of sucrose solution.

In humans, bioavailability of NAC is very low (4 - 10 %) yet may exhibit toxic effects (Borgstrom *et al.*, 1986; Olsson *et al.*, 1988; Kanter, 2006), especially when administered orally. Therefore, a very high concentration (1 µmol per insect) was chosen for caterpillar feeding (four times higher than the highest BSO concentration; roughly the maximum concentration achievable when dissolved in water). NAC (500 mM) was dissolved in 2 % sucrose and 2 µl were directly fed to the caterpillars with a pipette (Fig. 2). After 24 h, larvae were dissected and midgut epithelia were analysed for GSH concentration.

4.2.4 Time course of GSH manipulation

BSO specifically inhibits the enzyme catalysing the last step in GSH biosynthesis (γ-glutamylcysteine synthase). To assess how long inhibition lasts after treatment of *S. littoralis*, a time course experiment was conducted. Larvae (L3) were starved overnight and then received BSO (in 2 % sucrose solution) at 225 nmol per insect or 2 % sucrose solution without BSO as controls (Fig. 2). Immediately after that, larvae were put on artificial diet. At 0, 12, 24, 48 and 72 h post-ingestion, six caterpillars of the BSO treatment and two control larvae were dissected and their midguts analysed for GSH. The same experiment was conducted with NAC, but the dose of NAC was 1 µmol per insect.

4.2.5 Bioassays

Three different kinds of bioassays were performed:

Bioassay I aimed at comparing the effects of structurally different isothiocyanates when consumed at the same concentration. In this bioassay, larval performance was measured on diet containing 2 or 3 $\mu mol*g^{-1}$ isothiocyanate. Benzyl-, hexyl- and 2-phenylethyl (PE) isothiocyanate were tested separately against a control diet containing no isothiocyanate. These isothiocyanates were chosen for several reasons. First, they represent different classes of isothiocyanates, aliphatic and aromatic (indolic isothiocyanates were excluded because of their chemical instability). Second, hexyl-ITC is the aliphatic isothiocyanate with the longest carbon chain (and thus the least volatile) available commercially. Third, PE- and benzyl-ITC are two naturally occurring isothiocyanates which might be encountered by generalist herbivores. Both additionally have been investigated thoroughly in human medical and expecially in cancer research. Isothiocyanates were added in 0.5 ml sunflower seed oil per 100 g diet. Control diet contained 0.5 ml oil only.

Diet was provided in cubes of approximately 1 g and renewed daily because isothiocyanates showed high evaporation rates from the diet (around 50 % loss of detectable, i.e. free isothiocyanate after 24- 48 h, data not shown). Caterpillars were kept in individual, lidded plastic cups with a volume of approximately 60 ml on a laboratory bench at ambient room temperature and light conditions.

Individual larval performance of thirty randomly assigned caterpillars per treatment was measured from the first day of L2 until pupation by means of weight gain and diet consumption (both determined daily). Day of pupation, maximum weight and pupation weight was recorded for each individual. Prepupal and pupal mortality were assessed. Control cubes from each dietary treatment were kept under the same conditions as the larvae to correct for water loss from the diet cubes.

Bioassay II was designed to test for a correlation between the amount of isothiocyanate consumed and the amount of glutathione conjugates excreted in the faeces. One isothiocyanate (PE-ITC) was tested at three different concentrations: 0, 1 and 3 $\mu mol*g^{-1}$. In this experiment, the fate of the isothiocyanate was followed to determine the detoxification capacity related to isothiocyanate intake. For this, faeces of individual caterpillars were analysed for free isothiocyanate and GSH metabolites as described below. GSH metabolites include the full GSH conjugates with isothiocyanates and their corresponding CysGly conjugates, which are derived from the full GSH conjugates.

For this bioassay, caterpillars were assayed from the first day of L3 until one day after reaching the L4. L3 was chosen because caterpillars are less susceptible to high isothiocyanate concentrations (3 $\mu mol*g^{-1}$ diet) at this stage. Water loss from diet was controlled for as described for bioassay I. Larvae were assigned randomly to one of three treatments, with twenty larvae per treatment. For each individual, diet consumption and weight gain were determined daily until one day after reaching L4. Likewise faeces were collected daily and pooled over the duration of the experiment as described below.

Bioassay III manipulated larval detoxification capacity by modifying GSH levels in the midgut tissue. To enhance the total GSH concentration in larval midgut epithelia, NAC was administered to caterpillars at high concentrations (1 µmol per insect) on a daily basis. A reduction in GSH concentration was achieved by administering BSO to caterpillars at 225 nmol per insect every other day. Both BSO and NAC were dissolved in 2 % sucrose solution (at 112.5 and 500 mM, respectively) and administered to each insect in a volume of 2 µl (Fig. 2). A control group of caterpillars received 2 µl of pure sucrose solution.

Ninety larvae of similar weights were starved overnight during moulting to L3. On their first day as an L3, caterpillars were randomly assigned to one of the three treatments and received their respective dosage of BSO, NAC or sucrose solution. Twenty larvae of each treatment were then reared on diet containing 3 µmol PE-ITC per gram diet (see Bioassay I). The other 10 caterpillars of each treatment were fed on control diet (see Bioassay I) to control for the effects of GSH manipulation without the influence of isothiocyanate.

Larval weight and diet consumption were determined daily (as above), and faeces were collected daily (as described below). The experiment lasted 7 days with faeces collection continued individually till the first day after moulting to L4 or until caterpillars had reached a weight of at least 200 mg. After the end of the experiment, faeces were extracted and free isothiocyanate as well as GSH metabolites were quantified as described below.

4.2.6 Faeces collection and extraction and quantification of isothiocyanate and isothiocyanate-derived metabolites

Faeces were collected into a glass vial containing 200 µl of 0.1 M citrate buffer, pH 3.4, supplemented with 75 nmol of the glutathione conjugate of 4-methylsulfinylbutyl isothiocyanate (4msob-GSH) as internal standard, and 200 µl of dichloromethane, supplemented with 50 nmol of phenyl cyanide (phenyl-CN) as internal standard. Both aqueous and organic phases were used in order to extract both GSH conjugates and non-conjugated isothiocyanates. Between collections, faeces were stored at -20 °C.

Mixtures of faeces, collection buffer and dichloromethane were extracted twice with 0.75 ml citrate buffer (total 1.5 ml) for 8 min on a paint shaker (1000 U/min on a Skandex SO-10M, Fluid Management Europe B.V., Sassenheim, The Netherlands). In between extractions, solids were pelleted with a centrifuge (4200 x*g*) and supernatants were combined. Faeces were then extracted twice with 0.75 ml dichloromethane (total 1.5 ml), and the combined organic phases were partitioned against the aqueous extract to remove any remaining free isothiocyanate from the aqueous phase. Aqueous extracts were then sterile-filtered (pore size0.45 µm) and analysed using an LC-MS/MS. Quantification of the GSH- and the CysGly conjugate of PE-ITC (see Fig. 3) was relative to the internal standard (4msob-GSH) which was first normalised against external standard curves of both PE-GSH (response factor = 0.73) and PE-CysGly (response factor = 0.35). Total conjugate always includes both PE-GSH and PE-CysGly.

The organic phases were extracted twice against 0.1 M NaOH to remove 4-ethylbenzoic acid, a constituent of the artificial diet interfering with quantification of benzyl-ITC by GC-MS. Finally, organic phases were dried over Na_2SO_4 and analysed using a GC-MS system.

Extracted faeces were weighed to obtain a fresh weight, and then dried at 80 °C to obtain a dry weight. The amounts of free isothiocyanate and GSH metabolites were expressed relative to each other, to faeces weight and to diet consumption for each individual caterpillar.

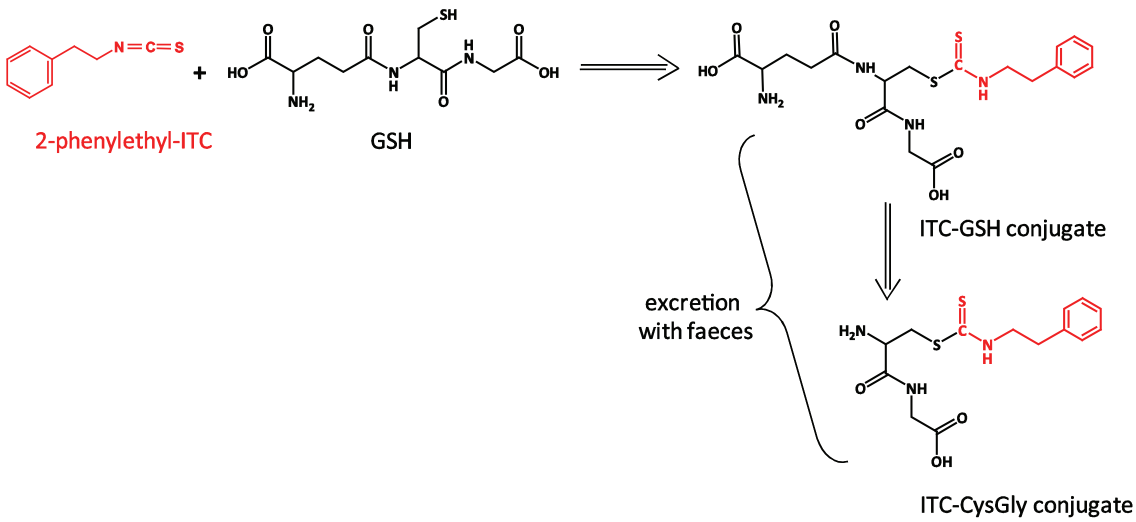


Fig. 3: Conjugation and metabolism of glutathione (GSH) conjugates of 2-phenylethyl isothiocyanate (PE-ITC) in humans.

The isothiocyanate moiety of PE-ITC becomes conjugated to the thiol residue of GSH. This process may be enzymatically catalysed by glutathione-*S*-transferases (GSTs) or spontaneous (see Chapter II) and is thought to take place in the gut epithelial cells (Traka & Mithen, 2009). The conjugates are then transported out of the cells (Traka & Mithen, 2009) and further metabolised to a cysteine-glycine conjugate (PE-CysGly). In this study, both GSH- and CysGly conjugates of PE-ITC were detected in larval faeces along with free PE-ITC.

4.2.7 Analysis of aqueous faeces extracts by LC-MS/MS

All HPLC analyses were carried out on Agilent 1200 series equipment (Agilent Technologies, Boeblingen, Germany). Separation was accomplished using an Agilent XDB-C18 column (50 mm x 4.6 mm, 1.8 µm; Agilent Technologies, Boeblingen, Germany) with a gradient of 0.05 % (v/v) formic acid in water (solvent A) and methanol (solvent B) following an elution profile of 0 - 0.5 min, 95 % A; 0.5 - 7.0 min, 95 - 0 % A; 7.0 - 8.0 min, 100 % B; 8.0 - 8.1 min, 100 - 5 % B; 8.1 - 12.0 min, 95 % A.

An API 3200 tandem mass spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with a Turbospray ion source was operated in the positive ionization mode. The instrument parameters were optimized by infusion experiments with pure standards (see Chemical syntheses). The ion spray voltage was maintained at 5500 eV, the turbo gas temperature was set at 700 °C, the nebulizing gas was set at 70 psi, curtain gas at 35 psi, heating gas at 60 psi and collision gas at 7 psi.

For analytes of interest, multiple reaction monitoring (MRM) was used to monitor parent ion to product ion conversion (Table 1). Analyst 1.5 software (Applied Biosystems) was used for data acquisition and processing.

Table 1: LC-MS/MS parameters for identification of GSH conjugates in aqueous faeces extracts of *S. littoralis*.

analyte abbreviation	analyte name	retention time [min]	precursor ion (m/z)	selected product ion (m/z)
PE-GSH	phenylethyl isothiocyanate-glutathione conjugate	6.2	471	105
PE-CysGly	phenylethyl isothiocyanate-cysteineglycine conjugate	5.9	342	105
4msob-GSH	4-methylsulfinylbutyl isothiocyanate-glutathione conjugate	4.1	485	179
PE-NAC	phenylethyl isothiocyanate-N-acetylcysteine conjugate	6.8	327	122

4.2.8 Analysis of organic faeces extracts by GC-MS

Separation, identification and quantification of free isothiocyanates in faeces extracts was done by GC-MS on an Agilent 6890 series gas chromatograph coupled with an Agilent 6973 Network series mass spectrometer. Separation was achieved on an SLB-5ms silica capillary column (30 m x 0.25 mm x 0.25 µm film; Supelco, Bellefonte, USA) using splitless injection at 200 °C, and the following temperature program: 40 °C for 3 min, a 10 °C min^{-1} ramp to 250 °C, a 60 °C min^{-1} ramp to 300 °C and a final 3 min hold. Quantification was done in single ion mode, using the following masses for quantification: m/z 103 – phenyl cyanide (internal standard), m/z 91 – PE-ITC. In single ion mode, PE-ITC had a response factor of 2.78 in relation to phenyl cyanide.

4.2.9 Chemical syntheses

GSH, CysGly and NAC conjugates of PE- and 4msob-ITC were chemically synthesised after Kassahun et al. (1997) with slight modifications. A quantity of 25 mg GSH, CysGly or NAC was dissolved in 0.5 ml 50 % (v/v) aqueous ethanol (pH 7.5) and mixed with 1 ml of 0.5 % (v/v) isothiocyanate solution in ethanol (5 mg isothiocyanate). The mixture was agitated under nitrogen on a vortex shaker over night. The identity of the reaction products was confirmed by LC-MS based on the *m/z* of fragments observed in MS^2 and MS^3 spectra. Structure of the NAC conjugate of PE-ITC (PE-NAC) was additionally confirmed by ^1H NMR.

4.2.10 Statistics

All statistical tests were carried out in R (The R-project, http://cran.r-project.org/index.html), except for the analysis of the hyperbolic curves in Bioassay II which were done in Sigmaplot (Sigmaplot 7.0, SPSS Inc). Experimental data representing more than two treatments were evaluated with ANOVAs (e.g. developmental parameters Bioassay I for 2 µmol ITC). Where there were only two treatments, a student's t-test was performed (e.g. developmental parameters Bioassay I for hexyl-ITC vs. control). The linear correlations between diet intake and weight gain in Bioassay I (3 µmol ITC) were calculated with a linear mixed effects model using weight gain, diet consumed and treatment as fixed factors, and "day" as a random factor.

4.3 Results

4.3.1 Isothiocyanates decrease larval performance with effects dependent on structure and concentration

Larvae of *Spodoptera littoralis* were reared on artificial diet containing three different isothiocyanates at 2 or 3 µmol*g^{-1} from the second instar (L2) until the moths had eclosed. All isothiocyanates caused differences in larval development compared to the no-isothiocyanate controls at 3 µmol*g^{-1} diet, leading either to high mortality or a significant delay in maturation (Table 2). The extent of mortality depended on the isothiocyanate (Fig. 4), with mortality on hexyl isothiocyanate (hexyl-ITC) reaching only 60 %, while mortality on benzyl- and 2-phenylethyl (PE)-ITC was 100 % and 97 % respectively (Table 2).

Table 2: Effects of isothiocyanates on larval development of *S. littoralis*. Caterpillars were reared on diet containing three different isothiocyanates at two concentrations. Where appropriate, means ± 1 standard error are given. Significantly different values ($p \leq 0.05$) are marked by different (superscribed) letters, with the same letters meaning no significant difference. n.a. = not applicable, measurements not performed due to almost complete mortality

2 µmol isothiocyanate/ g diet	mortality [%]	mean amount of diet consumed [mg]	mean weight gain [mg]	mean day of pupation	mean weight at pupation	mean maximum weight
control	20	1924 ± 358.8^{a}	469 ± 29.5^{a}	16.8^{a}	631.6 ± 44.8^{ab}	799.8 ± 28.1^{a}
benzyl-ITC	15	2474 ± 120.8^{a}	513 ± 31.6^{a}	17.4^{a}	515.96 ± 63.5^{a}	706.8 ± 27.5^{a}
hexyl-ITC	20	2248 ± 147.6^{a}	572 ± 47.4^{a}	17.3^{a}	709.3 ± 71.4^{ab}	876.6 ± 50.9^{a}
PE-ITC	50	2058 ± 200.8^{a}	620 ± 79.2^{a}	18.3^{a}	805.3 ± 86.1^{b}	896.7 ± 65.0^{a}

3 µmol isothiocyanate/ g diet	mortality [%]	mean amount of diet consumed [mg]	mean weight gain [mg]	mean day of pupation	mean weight at pupation	mean maximum weight
control	26.7	2003 ± 173.6^{a}	868 ± 72.6^{a}	16.7^{a}	923.7^{a}	1021.3^{a}
benzyl-ITC	100.0	n.a.	n.a.	n.a.	n.a.	n.a.
hexyl-ITC	60.0	1602 ± 211.1^{a}	530 ± 74.4^{b}	21.9^{b}	799.3^{a}	833.2^{b}
PE-ITC	96.7	n.a.	n.a.	n.a.	n.a.	n.a.

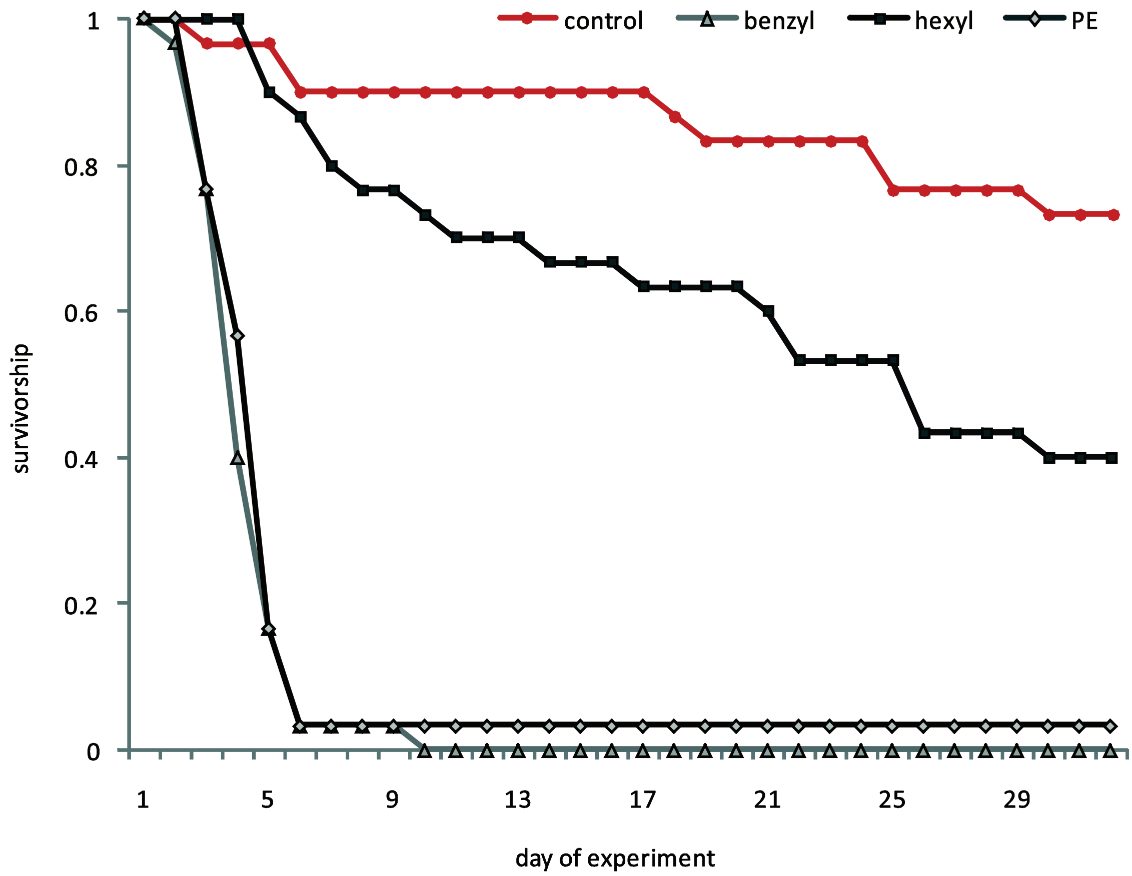


Fig. 4: Survivorship in caterpillars reared on artificial diet containing various isothiocyanates at 3 µmol*g^{-1}. Caterpillars were fed on artificial diet containing benzyl-, hexyl- or PE-ITC at 3 µmol*g^{-1} from L2 until pupation. Control caterpillars received the same diet without any isothiocyanate. Survivorship was determined daily and is given as proportion of surviving caterpillars relative to the number of caterpillars at the beginning of the experiment. The graph does not distinguish between larval and pupal mortality.

While mortality increased gradually in larvae feeding on control or hexyl-ITC diet, mortality on benzyl- and PE-ITC diets increased sharply within the first five days, reaching the final values within 10 days after starting the experiment. Concentrations of 3 µmol*g^{-1} isothiocyanate in the diet also caused other changes in larval performance (Table 2, Fig. 5A), but because of the high mortality on benzyl-ITC and PE-ITC diets, these changes were most apparent for the hexyl-ITC diet. Caterpillars reared on hexyl-ITC diet gained significantly less weight ($p = 0.002$), reached a significantly lower mean maximum weight ($p = 0.016$) and had a lower pupal weight than the controlslarvae on control diet (Table 2). Fewer caterpillars reached the pupal stage on hexyl-ITC, and the average date of pupation was significantly delayed in comparison to control larvae (Fig. 5B).

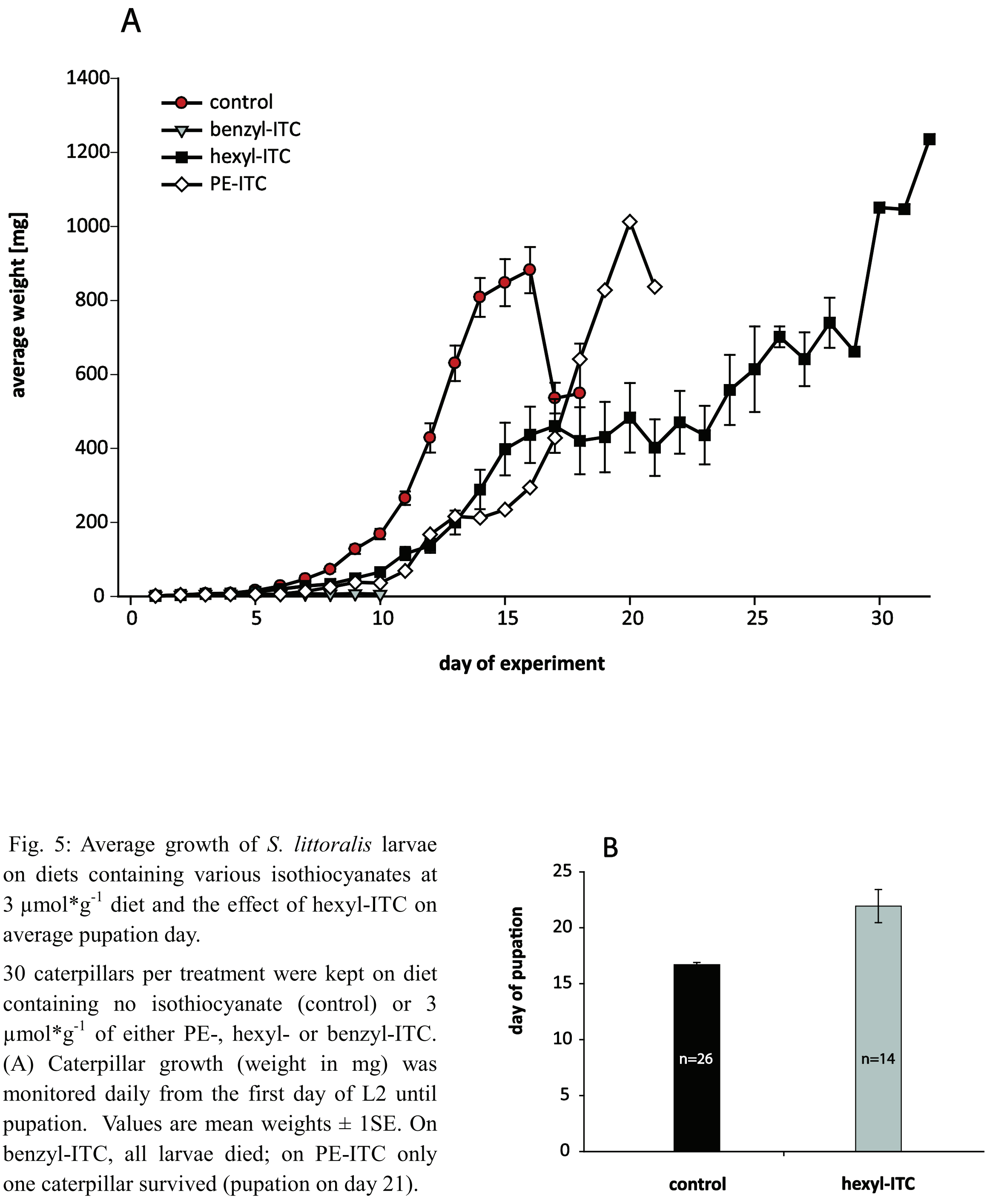


Fig. 5: Average growth of *S. littoralis* larvae on diets containing various isothiocyanates at 3 µmol*g^{-1} diet and the effect of hexyl-ITC on average pupation day.

30 caterpillars per treatment were kept on diet containing no isothiocyanate (control) or 3 µmol*g^{-1} of either PE-, hexyl- or benzyl-ITC. (A) Caterpillar growth (weight in mg) was monitored daily from the first day of L2 until pupation. Values are mean weights ± 1SE. On benzyl-ITC, all larvae died; on PE-ITC only one caterpillar survived (pupation on day 21).
(B) Pupation was reached by control caterpillars on day 17 on average. Caterpillars on hexyl-ITC reached pupation five days later on day 22. Of 30 larvae per treatment starting the experiment, 26 reached pupation on control diet, but only 14 reached pupation on diet with 3 µmol*g^{-1} hexyl-ITC.

Weight gain was positively correlated to intake of diet (Fig. 6) for both isothiocyanate-feeding and control larvae. Intake of caterpillars on the hexyl-ITC diet was not significantly different from that of caterpillars on the control diet (Table 2), yet control larvae gained more weight from the same amount of ingested diet (Fig. 6). The amount of diet consumed significantly influenced larval weight gain ($t = 20.4$, $p < 0.001$), but this was independent of the kind of diet ($t = -0.47$, $p = 0.64$). However, larval weight gain was significantly influenced by how much of which diet was consumed (interaction of diet and treatment). It thus made no difference which kind of diet was ingested when caterpillars consumed small amounts of diet. But when consuming larger amounts, caterpillars on the hexyl-ITC diet gained less weight compared to larvae on the control diet ($t = -2.6$, $p < 0.01$).

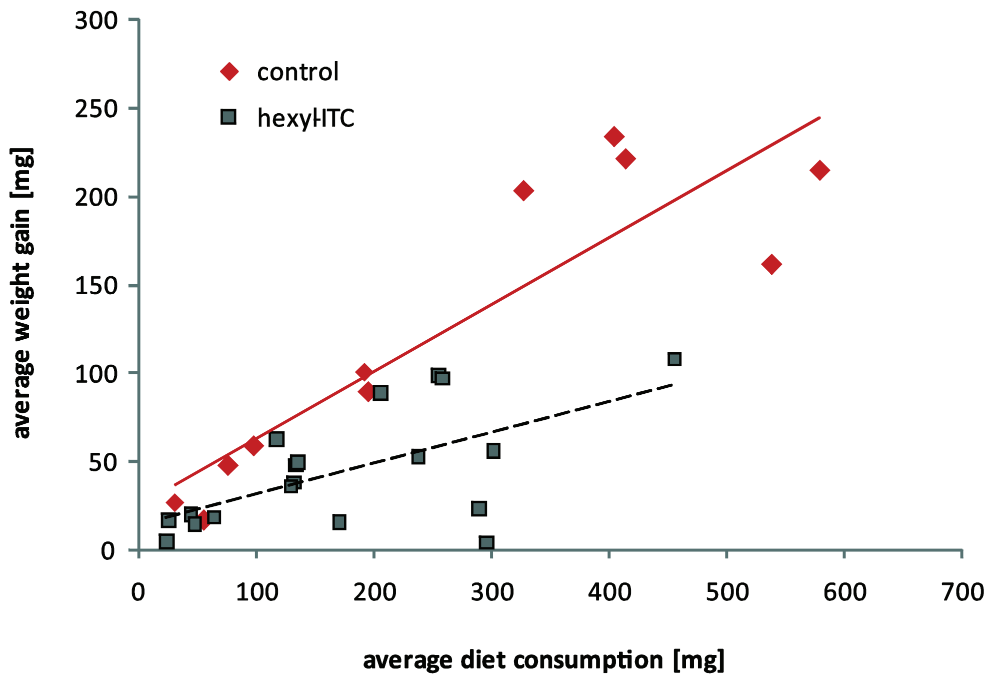


Fig. 6: Correlation between average daily weight gain and average daily diet consumption in *S. littoralis* larvae feeding on diet containing 3 µmol hexyl-ITC $*g^{-1}$ diet or no isothiocyanate control diet.

For 30 caterpillars per treatment, average weight gain [mg] is plotted in relation to average diet consumption [mg]. Data were collected daily from day 7 of the experiment until the onset of pupation; values represent means for every day of data acquisition. R^2 of control regression line = 0.799, R^2 of hexyl-ITC regression line = 0.3695. Weight gain is positively correlated to diet consumption ($p << 0.001$, $t = 20.4$). Weight gain depends on the kind of diet consumed (control or ITC) (mixed effects model, $p < 0.01$, $t = -2.6$).

When caterpillars were raised on diets containing 2 µmol*g^{-1} isothiocyanate, differences in mortality and performance were observed, but the effects were less striking than at 3 µmol*g^{-1} isothiocyanate. Mortality was increased only for larvae feeding on PE-ITC. Although developmental parameters differed between treatments, no significant effects were found except for average pupation date (Table 2). During early larval development, isothiocyanates significantly reduced weight gain of caterpillars, while differences were less pronounced later in development (Supplemental Fig. S1). Taken together, our data show that isothiocyanates, mixed into an artificial diet, had a negative impact on survivorship and development of *S. littoralis* with effects depending on concentration and structure The apparent order of toxicity is 2-phenylethyl > benzyl > hexyl. Because the effects of PE-ITC were most pronounced, subsequent experiments were conducted with this compound.

4.3.2 Formation of isothiocyanate-GSH conjugates is limited at higher levels of isothiocyanate ingestion

Experiments with PE-ITC assessed the dose-dependent effects of this substance on larval development, and its metabolic fate. As already observed in the previous experiment, larval development was strongly affected by the presence of 3 µmol*g^{-1} PE-ITC in the diet (Fig. 7) in terms of weight gain, mortality and development time (time between L3 and L4).

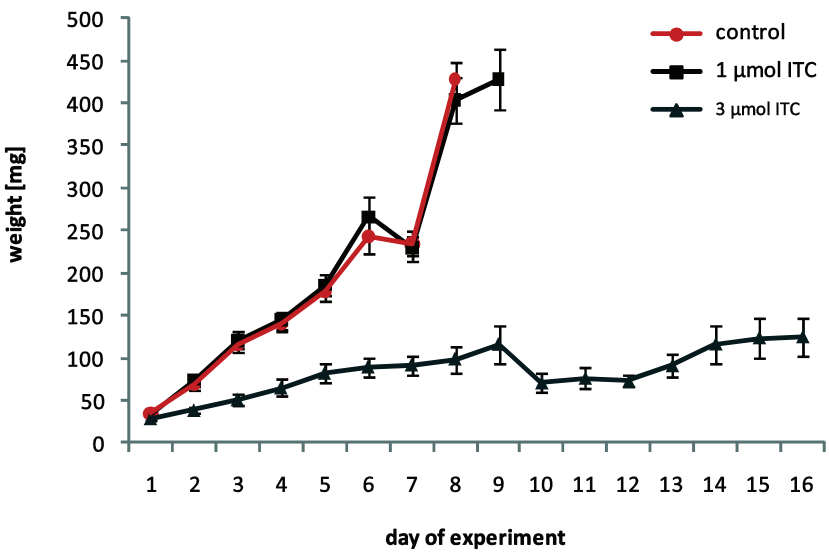


Fig. 7: Larval performance of *S. littoralis* on artificial diet containing 0, 1 or 3 µmol*g^{-1} PE-ITC.

Caterpillars were kept on one of the three diets from the first day of L3 until the second day of L4. On diet with 1 µmol*g^{-1} PE-ITC survival was 100%, the same as for control caterpillars. On 3 µmol ITC *g^{-1} diet, survival was reduced to 70% at the end of the experiment (large triangles stand for dead individuals).

However, there was no detectable difference in larval weight gain or development time between controls and caterpillars feeding on 1 µmol PE-ITC $*g^{-1}$ diet, so the effect of this isothiocyanate is clearly dose-dependent. In the faeces of caterpillars feeding on PE-ITC, the free isothiocyanate and its GSH metabolites, PE-GSH and PE-CysGly, were detected (Table 1 and Fig. 3). The amounts of these compounds differed depending on the isothiocyanate concentration in the diet (Fig. 8). When plotting the amount of PE-ITC ingested per larva against the amount of GSH metabolites excreted in the faeces (Fig. 8A), the curves are non-linear, and best described by hyperbolic functions (1 µmol: $R^2 = 0.287$, $p = 0.02$, 3 µmol: $R^2 = 0.584$, $p = 0.001$). Thus, conjugate formation at higher levels of isothiocyanate ingestion is limited.

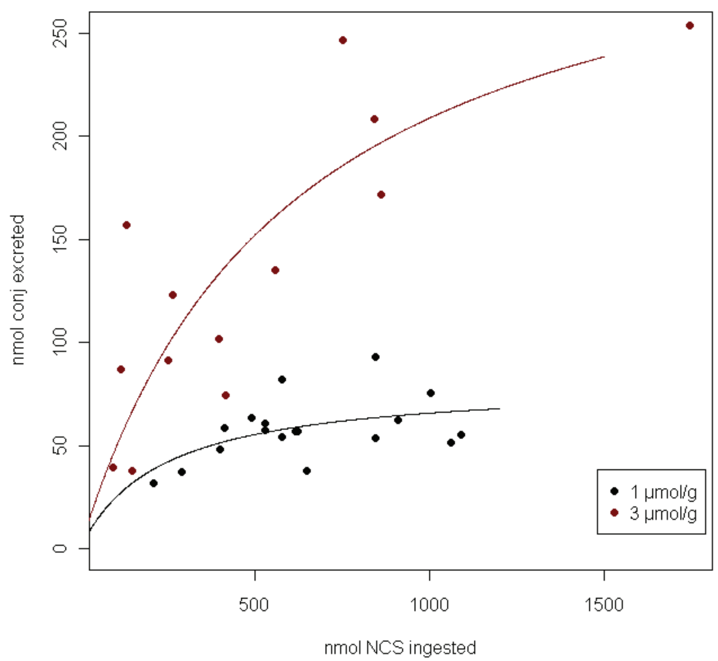
The relationship of free PE-ITC excreted with the amount ingested also follows a hyperbolic function when caterpillars ingest 3 µmol$*g^{-1}$ PE-ITC diet. However, this relationship is linear when caterpillars fed on diet containing 1 µmol PE-ITC $*g^{-1}$ (Fig. 8B; $R^2 = 0.586$, $p << 0.01$).

Overall, the amounts of free isothiocyanates excreted were higher than those of GSH metabolites in the faeces, showing that only part of the ingested compound was metabolised. Comparing diet with a low concentration of PE-ITC (1 µmol$*g^{-1}$) to that with a high concentration (3 µmol$*g^{-1}$), the excretion of GSH conjugates appeared to increase correspondingly, but excretion of free PE-ITC did not (Fig. 8B, Supplementary Fig. S2). This suggests that at high isothiocyanate concentrations, a greater proportion of this toxin has another metabolic fate besides simple excretion and conjugation with GSH.

4.3.3 Glutathione content of midgut epithelial cells is depleted by isothiocyanate induction

The GSH content of midgut epithelial cells of *S. littoralis* caterpillars averaged at approximately 24 nmol$*mg^{-1}$ protein (Fig. 9). When caterpillars received 3 µmol$*g^{-1}$ PE-ITC, GSH was significantly reduced after 24 h to approximately 8 nmol GSH $*mg^{-1}$ protein ($p << 0.0001$; Fig. 9A). Thus PE-ITC can act as GSH depleting agent. Inside the gut lumen, no GSH was detectable. Analysis of the food bolus (inside the peritrophic membrane) demonstrated that there is no GSH present, regardless of whether caterpillars had fed on plant material or artificial diet (data not shown).

A



B

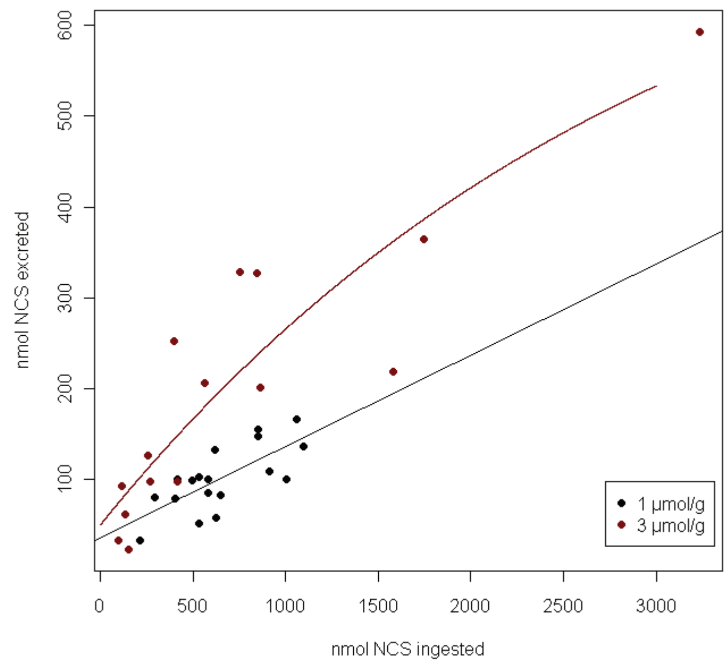


Fig. 8: Correlations between the amount of PE-ITC ingested by *S. littoralis* and the amount of GSH metabolites or free ITC excreted. Twenty L3 larvae were kept on diet containing 1 or 3 µmol PE-ITC $*g^{-1}$ for the duration of one larval instar.

(A) The correlations between PE-ITC ingestion and GSH metabolites fit hyperbolic curves and are significant for both ITC concentrations.

(B) The correlation between PE-ITC and free ITC is linear at 1 $\mu mol*g^{-1}$ diet and hyperbolic at 3 $\mu mol*g^{-1}$ diet

4.3.4 GSH levels can be influenced pharmacologically

To investigate the effect of GSH supply on isothiocyanate detoxification, we developed methods to manipulate the internal GSH level of *S. littoralis* larvae. Depletion was achieved by using L-buthionine sulfoximine (BSO), which irreversibly inhibits the last enzymatic step in GSH biosynthesis. Larvae which had received 225 nmol BSO per insect experienced a significant decrease in GSH content to 12 nmol GSH $*mg^{-1}$ protein ($p << 0.01$). GSH elevation was accomplished with *N*-acetyl cysteine (NAC). L3 caterpillars, which had received 1 µmol NAC per insect, had a significantly increased GSH concentration in their midgut epithelial cells from 34 to 47 nmol GSH $*mg^{-1}$ protein ($p = 0.008$). The difference in cellular GSH content between BSO and NAC larvae was also significant ($p << 0.01$).

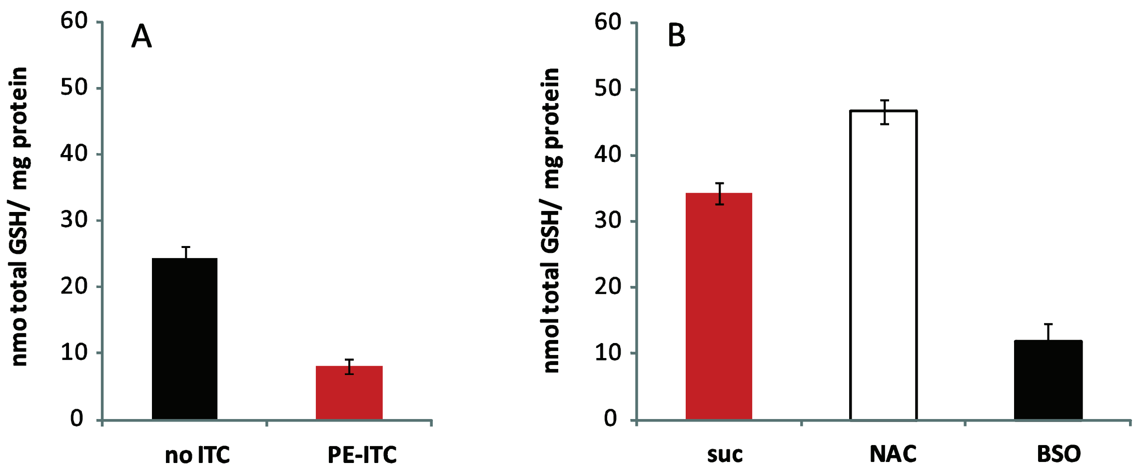


Fig. 9: Effect of PE-ITC, NAC and BSO on GSH concentration in *S. littoralis* midgut epithelia.

A) After 24 h on diet with 3 µmol PE-ITC $*g^{-1}$, the amount of GSH $*mg^{-1}$ protein was significantly reduced with $p << 0.01$ (Welch Two Sample t-test).

B) Caterpillars received 1 µmol NAC per insect, 225 nmol BSO in 2% sucrose per insect, or 2% sucrose solution as a control. After feeding on artificial diet for 24 h, NAC treatment significantly increased GSH content compared to control ($p = 0.008$) and BSO-treated larvae ($p < 0.0001$). BSO significantly reduced GSH content in comparison to sucrose ($p = 0.0002$). Statistics are based on a One-way-ANOVA and a Tukey post-hoc test.

To determine the duration of these altered GSH levels, a time course experiment was performed using 100 or 225 nmol BSO per insect. Twelve hours post-treatment GSH levels were reduced to appr. 25 % compared to control levels at both BSO concentrations (Fig. 10).

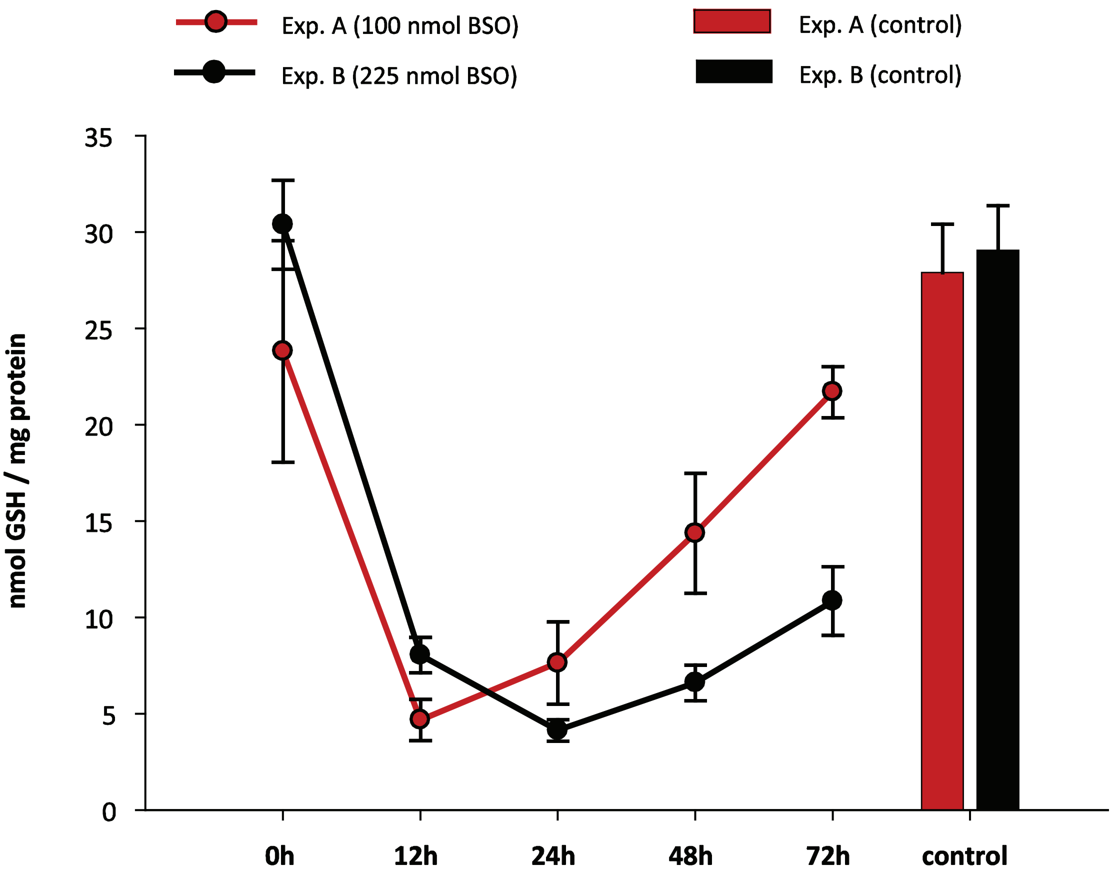


Fig. 10: Dose-dependent effects of BSO on total GSH content in *S. littoralis* midgut epithelia over a time course.

L3 larvae received BSO (in 2 % sucrose solution) once at 100 or 225 nmol per insect and were followed for 72 h. The GSH reduction for 225 nmol BSO is significant at all time points compared to 0 h. The reduction for 100 nmol BSO is significant at all times except 72 h.

BSO concentration did not have an impact on the extent of GSH depletion but had an influence on the duration of the effect. When treated with 225 nmol BSO per insect, GSH was still fully reduced at 48 h, but at that time, GSH levels had recovered about half of their control values in caterpillars which had received only 100 nmol BSO. Thus a 225 nmol-dose should suffice to deplete GSH by 75% for 48 h. An analogous time course experiment with NAC yielded highly variable results (not shown). Since it is known that supplemented NAC has a short half life time *in vivo* (Borgstrom *et al.*, 1986; Olsson *et al.*, 1988), NAC was administered daily at 1 µmol per insect.

4.3.5 Manipulation of glutathione concentration has an impact on larval detoxification capacity

Since glutathione seems to play a pivotal role in the processing of isothiocyanates in *S. littoralis*, and formation of PE-ITC depletes GSH in the midgut epithelia of L3 larvae (see previous section, Fig. 9A), manipulating the pool of available glutathione might change detoxification capacity. We altered the glutathione levels with NAC and BSO, while feeding larvae on a diet containing 3 µmol*g^{-1} PE-ITC. Caterpillars whose GSH levels had been increased by NAC grew best on the isothiocyanate-containing diet while caterpillars whose GSH levels had been decreased by BSO showed the least growth (Fig. 11A). Thus GSH supply seems to significantly affect larval performance presumably by influencing the rate of isothiocyanate processing. On non-isothiocyanate diet, control and BSO-treated caterpillars grew almost equally well but NAC-treated larvae performed less well (Fig. 11B). This indicates that the good performance of NAC-treated larvae on isothiocyanate diet might be even better if not for the negative side effects, even more because with isothiocyanate challenge these larvae did better than without. If either NAC or BSO treatment influences isothiocyanate processing capacity, this should be reflected in the amounts of GSH metabolites (GSH and CysGly conjugates, Table 1, Fig. 3) in the faeces.

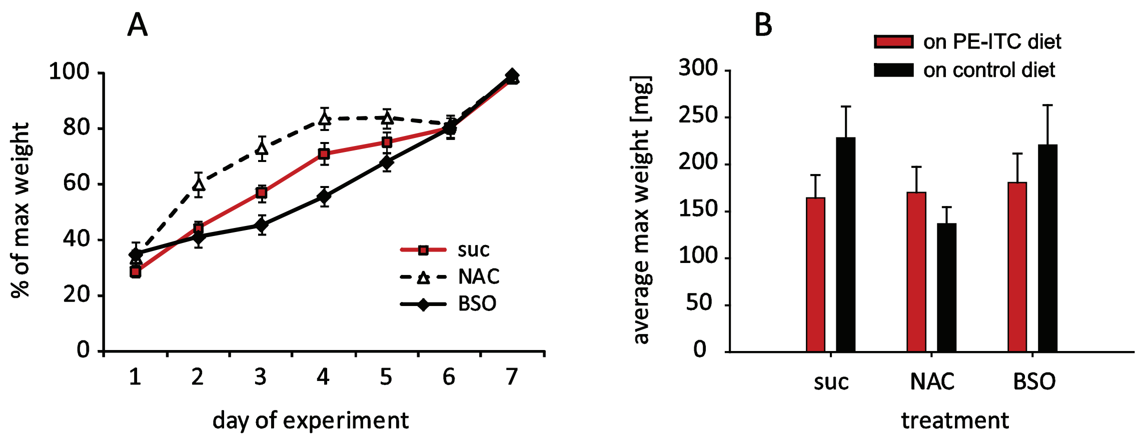


Fig. 11: Effect of manipulating GSH with NAC and BSO on growth of caterpillars feeding PE-ITC.

BSO treatment was 225 nmol in 2% sucrose every other day. NAC treatment was 1 µmol in 2% sucrose daily. The experiment was carried out for 7 days with weight measured daily from day 1 of L3 to day 2 of L4. The majority of larvae moulted on day 6.
(A) Comparison of larval performance on PE-ITC diet as % of maximum weight.
(B) Average maximum weight [mg] of the caterpillars on PE-ITC and control diets.

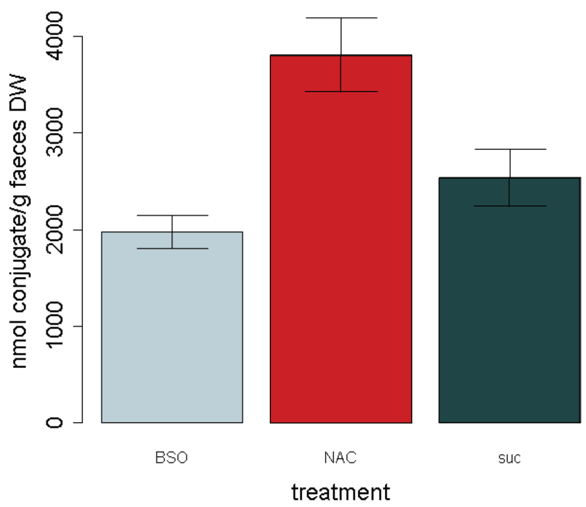


Fig. 12: Effect of manipulation of *S. littoralis* GSH content with NAC and BSO on the level of isothiocyanate metabolites in faeces.

Treatments were the same as in Fig. 11. Larvae receiving control (2% sucrose, "suc") or BSO treatment contained significantly less conjugates per gram faeces than NAC larvae (One-Way-ANOVA, Tukey post-hoc test, both p_{BSO} and $p_{suc} < 0.01$). The difference between control and BSO caterpillars was not significant ($p = 0.37$).

Indeed faeces of NAC-treated larvae contained significantly more isothiocyanate conjugates than those of either control (suc, $p = 0.009$, One Way ANOVA) or BSO-treated caterpillars ($p << 0.01$, One Way ANOVA) (Fig. 12). Control larvae excreted more conjugates than BSO larvae, but the difference was not significant ($p = 0.37$, One Way ANOVA). Thus GSH content had a direct effect on the amounts of GSH metabolites in the faeces.

4.4 Discussion

Isothiocyanates, usually considered the most toxic group of glucosinolate break-down products, constitute a direct defence mechanism against herbivorous insects and other enemies found in plants belonging to the order Brassicales (Chew, 1988; Lambrix *et al.*, 2001; Wittstock *et al.*, 2003; Halkier & Gershenzon, 2006). Several studies have already been conducted to assess the direct effects of isolated isothiocyanates on insects (Wadleigh & Yu, 1988; El Sayed *et al.*, 1996; Borek *et al.*, 1998). Here we compare the effects of three different isothiocyanates on larvae of the generalist insect herbivore *Spodoptera littoralis*, and investigate the formation of isothiocyanate-glutathione (GSH) conjugates, a presumptive route for isothiocyanate detoxification. The rate of formation of GSH conjugates is reduced when the isothiocyanate is high and when GSH supply is low.

4.4.1 Isothiocyanate toxicity is manifest in increased mortality, extended development time and reduced diet consumption

The toxicity of isothiocyanates (ITC) to *S. littoralis* is starkly illustrated by the nearly 100% mortality suffered on diets of benzyl- and PE-ITC (Fig. 4 & Table 2) at 3 µmol*g^{-1}. However, at 2 µmol isothiocanate *g^{-1}, mortality on benzyl-ITC was not different from that on control diets, while PE-ITC still caused significant mortality (Table 2). The dose-dependency of isothiocyanate toxicity has already been documented for other insect species (Borek *et al.*, 1998; Li *et al.*, 2000). However, in prior studies it is not always clear if measurements of isothiocyanate concentration in diet took into account the volatility of these substances. In all of our feeding assays, isothiocyanate-containing diet was exchanged daily, because preliminary tests had shown that isothiocyanate concentration in 1 g diet cubes decreased by up to 50% within 48 h, probably due to volatilisation since no traces of isothiocyanate conjugates were found. Moreover, prior studies often used allyl isothiocyanate which is even more volatile than the isothiocyanates used herein.

Isothiocyanate toxicity also depended on the structure of the molecule. Taking data at the two tested concentrations together, PE-ITC had the greatest mortality, followed by benzyl-ITC and then hexyl-ITC. A study by Wadleigh and Yu (1988), comparing the toxicity of allyl-, benzyl- and PE-ITC to *S. frugiperda*, also found that the aromatic isothiocyanates were more toxic than the aliphatic isothiocyanates and that the actual toxicity (LC_{50} values) was similar to that shown here for *S. littoralis*.

Borek *et al.* (1998) showed that isothiocyanate toxicity to eggs of the black vine weevil (*Otiorhynchus sulcatus*) increases with molecular weight, which is also consistent with the results presented here. However, it is not clear what parameter- chemical reactivity, polarity, solubility or volatility- is most important in determining toxicity.

Isothiocyanate toxicity was also reflected in extended development times. Even when *S. littoralis* larvae survived until pupation on 3 µmol*g^{-1} ITC, they were delayed in reaching the pupal stage (Figure 5B, Table 2). Isothiocyanates have also been shown to cause prolonged development times in previous studies (Agrawal & Kurashige, 2003; Burow *et al.*, 2006; Ulmer & Dosdall, 2006). Increasing larval development times will benefit a potential host plant, since it extends the exposure time of caterpillars during this vulnerable life stage to predators, pathogens and abiotic stresses (Brattsten, 1986), and could be regarded a good defence strategy of plants.

Delays in pupation (or any larval moult) arise because moulting only occurs after larvae have reached a certain body size or nutritional status (Gullan & Cranston, 1996; Gillott, 2005). At 3 µmol isothiocyanate *g^{-1} diet, the average maximum weight of caterpillars on isothiocyanate diet was significantly lower than that of control larvae (Table 2). In addition, the larvae reared on isothiocyanate consumed less diet over all (Fig. 6), suggesting that isothiocyanates may have had a feeding deterrent effect in our bioassays. Correlations of diet consumption and larval weight reveal that larvae had to consume more isothiocyanate-containing diet than control diet to achieve the same weight gain, indicating that this toxin could reduce the nutritional value of the diet. Feeding on a diet of reduced nutritional quality could account for the delays in larval development.

Although the data presented here and in other studies (Wadleigh & Yu, 1988; Borek *et al.*, 1998; Agrawal & Kurashige, 2003; Ulmer & Dosdall, 2006) demonstrate the ability of isolated isothiocyanates to impact the development and survival of generalist caterpillars, it is important to keep in mind that plants do not contain preformed isothiocyanates as toxins but produce them during feeding damage due to myrosinase-catalyzed hydrolysis of glucosinolates. Thus, studies with isolated isothiocyanates are imperfect models of glucosinolates as plant defence strategies. In addition, under natural conditions caterpillars would rarely be challenged by a single isothiocyanate since plants of the Brassicales typically contain a spectrum of different glucosinolates (Fahey *et al.*, 2001; Brown *et al.*, 2003) which often belong to more than one structural family (i.e. indolic, aliphatic or aromatic glucosinolates). Thus, further experiments should strive to test the effects of mixtures of isothiocyanates (or intact glucosinolates) on generalist herbivores.

4.4.2 Isothiocyanates detoxification depends on GSH availability

Whereas specialist herbivores have developed diverse strategies to avoid exposure to toxic isothiocyanates on glucosinolate hydrolysis (Ratzka *et al.*, 2002; Wittstock *et al.*, 2004; Muller & Wittstock, 2005), generalist insects are often believed to detoxify isothiocyanates by conjugation with GSH (Yu, 1983; Wadleigh & Yu, 1988; Francis *et al.*, 2005). The success of such a strategy may depend on an adequate supply of GSH.

GSH conjugation with isothiocyanates might conceivably occur in the glut lumen before these toxins are absorbed. The reaction would be chemically favoured at the highly basic pH, shown to occur in the gut lumina of many herbivorous insects ((Appel & Martin, 1990; Dow, 1992); in *S. littoralis*- pH 8 - 10, L. Ping, pers. communication). However, in this study no GSH was detectable in the gut contents of *S. littoralis* caterpillars (peritrophic membrane plus food bolus). Thus we assume that isothiocyanate conjugation in *S. littoralis* may occur in the gut epithelial cells instead. GSH is an intracellular thiol that is not frequently exported from cells (Griffith, 1999). Furthermore, in humans isothiocyanate conjugation is also thought to occur in the gut epithelial cells (Traka & Mithen, 2009).

The first hint that isothiocyanate detoxification capacity might be dependent on GSH levels in *S. littoralis* larvae is provided by the our data, because PE-ITC fed to these caterpillars led to a significant decrease in cellular GSH after 24 h (Fig. 9A). Isothiocyanates also cause a strong depletion of GSH levels in human and rat cell lines (Zhang, 2000; Ye & Zhang, 2001; Zhang, 2001). But in humans, after initial reduction of GSH pools, GSH biosynthesis is induced quickly after incubation with isothiocyanate and a significant increase in cellular GSH is seen in comparison to normal levels (Ye & Zhang, 2001; Zhang *et al.*, 2005). However, in our *S. littoralis* larvae, GSH pools were still depleted 24 h post isothiocyanate treatment, indicating that GSH supply could be limiting for isothiocyanate processing.

When *S. littoralis* caterpillars were reared on different concentrations of PE-ITC, toxicity was dose-dependent (Fig. 7). In addition, the excretion of free PE-ITC and its GSH metabolites in the faeces was dose-dependent: the more isothiocyanate larvae ingest, the more free isothiocyanate and GSH metabolites are excreted (Fig. 8). Yet, for the GSH conjugates this correlation is not linear (Figs. 8, S2) but follows a hyperbolic function, demonstrating that conjugate formation is limited at higher levels of isothiocyanate intake. The excretion of free isothiocyanate relative to isothiocyanate ingested also follows a hyperbolic relationship at higher dietary levels of isothiocyanates, suggesting that elimination of free isothiocyanate is also limited when isothiocyanate intake is high.

Thus neither isothiocyanate conjugation nor isothiocyanate release keep up with isothiocyanate intake at high ingestion rates, which is likely responsible for the increased mortality and reduced growth observed under these conditions.

A similar effect of PE-ITC was observed in rats where pharmacokinetics of isothiocyanate metabolism were studied (Ji *et al.*, 2005). Investigation of the temporal progress of PE-ITC clearance showed that clearance decreased significantly with increasing isothiocyanate doses. The authors speculate that the isothiocyanate elimination is limited by availability of glutathione-*S*-transferases (GSTs) and other enzymes involved in mercapturic acid formation. Consistent results were also found by other studies (Hudson *et al.*, 2005; Hanlon *et al.*, 2008). Despite the apparent importance of isothiocyanate-GSH conjugate formation for isothiocyanate detoxification in *S. littoralis*, GSTs were not found to be involved in this process (see Chapter II).

Our manipulation of GSH content in *S. littoralis* (Fig. 11A) also demonstrated the influence of GSH supply on isothiocyanate detoxification. When GSH levels were pharmacologically reduced with BSO, caterpillars feeding on PE-ITC diet excreted less GSH metabolites per gram faeces and were diminished in growth compared to controls. These results are consistent with studies in insects, rodents and mammalian cell lines which have shown that BSO can diminish detoxification capacity (Masuh *et al.*, 1996; Paes *et al.*, 2001; Hsu *et al.*, 2002; Andringa *et al.*, 2006). Moreover, it was shown that BSO and isothiocyanate together reduce GSH pools more than either compound alone (Xu & Thornalley, 2001; Zhang *et al.*, 2005). When GSH levels were pharmacologically enhanced with NAC, larvae excreted more GSH metabolites per gram faeces than either control or BSO-treated caterpillars, and experienced an increase in growth. The beneficial effects of increased GSH levels on isothiocyanate processing can be fully appreciated, when the poor performance of NAC-treated larvae (lowest mean weight gain, highest mortality in comparison to the other treatments) without any challenge by isothiocyanates is regarded.

GSH is an essential metabolite in living organisms that functions not only in detoxifying isothiocyanates and other xenobiotics, but in many other processes such as redox control, protection against oxidative stress, protein synthesis, DNA synthesis and repair, amino acid transport and functioning of the immune system (Griffith, 1999; Dickinson & Forman, 2002; Estrela *et al.*, 2006). Hence, depletion of GSH in *S. littoralis* by a dietary compound may reduce physiological vigour in a number of ways, making caterpillars more susceptible to predation, parasitism or disease. Our experiments were performed in a laboratory setting on an artificial diet with a complete nutrient supply and without other stressors like predators, parasitoids or extremes of light and temperature. Under field conditions, when *S. littoralis* larvae face more variable nutritional, biotic and abiotic stresses, GSH levels may limit isothiocyanate processing even more.

Apparently, detoxification of isothiocyanates by conjugation with GSH is an effective strategy, since it is applied by different species of generalist lepidopteran herbivores (see Chapter I). Yet, is it also an efficient strategy? Although it seems to suffice for generalists under most conditions, it certainly is expensive, considering the loss of nitrogen and the importance of GSH in other processes. Also, as demonstrated here, GSH stores may become depleted with high risk of damage to the herbivore. To further elucidate the precise mechanisms behind isothiocyanate conjugation with GSH, experiments could include insects under oxidative or nutrient stress, where GSH availability may be critical. Other trials could use insects of different larval stages with presumably differing detoxification capacities, or generalists with a broad as opposed to a narrow host range. Such trials can also shed light on the resilience of this generalist detoxification strategy and on how far herbivores may exhaust it before hitting its limits.

5 General discussion

The activated defence system of plants in the order Brassicales, the glucosinolate myrosinase system, has been studied thoroughly both from the plant and the insect side. While lots of progress has been made on all frontiers, the question of how glucosinolate hydrolysis products are metabolized in generalist insect herbivores has long remained unanswered. Specialized insect feeders have been shown to use particular pathways to circumvent glucosinolate toxicity, but little is known about generalist herbivores, although many generalist lepidopterans are important pests on agronomically important Brassicaceae. Understanding how glucosinolate hydrolysis products act on generalist herbivores and how they are metabolized can lead to new routes of pest management.

In this thesis, several aspects of glucosinolate hydrolysis product processing in generalist lepidopteran caterpillars were investigated. As described in Chapter I, isothiocyanates are excreted as glutathione (GSH) - and cysteinylglycine (CysGly) conjugates, and as free isothiocyanates in several special of lepidopteran generalists. These conjugates are found in faeces, regardless of the parent glucosinolate structure or the species of herbivore involved. This is the first time that such metabolites have been described in the faeces of generalist insect herbivores. The fact that conjugates from all tested structures can be found in all species of herbivores investigated suggests that conjugation of isothiocyanates with GSH is a common processing strategy in lepidopteran generalists that leads to detoxification. Whether glutathione-*S*-transferases (GSTs) are involved in the conjugation of isothiocyanate with GSH was addressed in Chapter II of the thesis. In *in vitro* enzyme assays with *Spodoptera littoralis* GSTs, enzyme activity above the background of spontaneous GSH-isothiocyanate condensation was not detected under physiologically realistic conditions, suggesting the formation of conjugates occurs via non-enzymatic processes. In Chapter III, the biological consequences of isothiocyanate consumption and processing were assessed and some of the parameters that influence conjugation capacity were investigated. It was shown that the availability of GSH plays an important role, much more important than previously thought if GSTs are not directly involved, as inferred from Chapter II. Based on these results, a new model of isothiocyanate metabolism in generalist insects was introduced.

Upon hydrolysis, glucosinolates can be broken down to a number of different products. The outcome of hydrolysis depends mainly on the presence of so-called specifier proteins, ferrous ions and the structure of the variable side chain. The hydrolysis products which have been studied most thoroughly are the isothiocyanates, and often the biological activity of a glucosinolate can be attributed to its corresponding isothiocyanate (Wittstock *et al.*, 2003). Hence, specialised insect herbivores try to avoid the formation of toxic isothiocyanates using different strategies. The adaptations to the glucosinolate myrosinase system of two major lepidopteran pests have been investigated thoroughly because these species have a considerable impact in agriculture. Both larvae of the small cabbage white *Pieris rapae* and the diamondback moth *Plutella xylostella* use protein-based mechanisms to prevent isothiocyanate formation. The nitrile specifier protein (NSP) of *P. rapae* redirects glucosinolate hydrolysis towards less toxic simple nitriles (Wittstock *et al.*, 2004), which have been shown to play an important role in the attraction of *P. rapae* parasitoids. So, the mechanism saving *P. rapae* from isothiocyanates at the same time attracts the caterpillars' enemies (Mumm *et al.*, 2008). In *P. xylostella*, a sulphatase removes the sulphate moiety from the glucosinolates, rendering them unsuitable substrates for myrosinases (Ratzka *et al.*, 2002). Both *P. rapae* and *P. xylostella* are susceptible to isothiocyanates if forced to feed on them (Li *et al.*, 2000; Agrawal & Kurashige, 2003), thus demonstrating that avoidance of isothiocyanate formation is an important strategy for feeding on glucosinolate-containing plants. Other herbivores avoid isothiocyanate formation by sequestering intact glucosinolates, some of them using them for their own defence (Muller *et al.*, 2001; Aliabadi *et al.*, 2002; Bridges *et al.*, 2002). But most of those are phloem feeders which do not trigger the mustard oil bomb (myrosinase-catalyzed hydrolysis of glucosinolates) by their normal feeding behaviour. An exception is the sawfly *Athalia rosae,* whose larvae chew on leaves like caterpillars, but sequester intact glucosinolates (Muller & Wittstock, 2005). The mechanism by which *A. rosae* avoids isothiocyanate toxicity is still unknown.

As lepidopteran herbivores abolish the spatial separation of glucosinolates and myrosinase by chewing the leaf material, they trigger the formation of hydrolysis products. In the absence of special adaptations, isothiocyanate production is a frequent outcome of generalist herbivore feeding. An exception is the locust *Schistocerca gregaria*, which produces a sulphatase activated only when this generalist feeds on glucosinolate-containing plants (Falk & Gershenzon, 2007). Like in *P. xylostella*, glucosinolates are desulphated and excreted. Nonetheless, when feeding on Brassicaceae most generalist insect herbivores are faced with isothiocyanates, whose biological activity against many different organisms has been demonstrated repeatedly. If evasion of toxic isothiocyanates is not possible, insects must be insensitive to these general poisons or detoxification must occur upon ingestion.

Yet so far, little was known about glucosinolate and isothiocyanate metabolism in generalist insects. Isothiocyanates were shown to induce GSTs in several species of insect herbivores (Yu, 1983; Wadleigh & Yu, 1988; Francis *et al.*, 2005). In GST enzyme assays, GSH concentration decreased upon addition of isothiocyanate, suggesting a GST-driven conjugation of isothiocyanates with GSH (Wadleigh & Yu, 1988). This is similar to what occurs in humans and other mammals, where isothiocyanates are metabolised by conjugation with GSH and excreted as mercapturic acids (Habig *et al.*, 1974). Other mammals have also been shown to conjugate isothiocyanates with GSH, although mercapturic acids are not always the final product of this metabolic pathway (Conaway *et al.*, 2002). In addition to isothiocyanate-GSH conjugates, there are other glucosinolate metabolites to look for in faeces of generalist herbivores, if one considers which compounds are excreted by specialist insect herbivores. For example, *P. rapae* excretes mostly nitriles (Wittstock *et al.*, 2004; Agerbirk *et al.*, 2006; Vergara *et al.*, 2006; Vergara *et al.*, 2007; Agerbirk *et al.*, 2010) and *P. xylostella* excretes desulfo-glucosinolates (Ratzka *et al.*, 2002).

When faeces of various lepidopteran generalist-feeding larvae were analysed for possible glucosinolate metabolites in the course of this thesis, GSH- and CysGly-isothiocyanate conjugates and free isothiocyanates were detected. Nitriles or desulfo-glucosinolates were not found, indicating that the tested species possess neither an NSP nor a glucosinolate sulphatase. The fact that both GSH- and CysGly-isothiocyanate conjugates are present in the faeces shows that insects perhaps possess a pathway similar to the mercapturic acid pathway, or a pathway that further metabolises isothiocyanates after their conjugation with GSH. As CysGly-isothiocyanate conjugates were also found after caterpillars had fed on artificial diets containing isothiocyanates (see Chapter III), they must be a larval product rather than a chemical metabolite of isothiocyanates. This is also supported by the absence of CysGly-conjugates in *in vitro* enzyme assays or in the isothiocyanate-containing diet itself. Hence, CysGly-conjugates are formed neither spontaneously in the diet nor by GST activity. In mammals, it is known that each step of the mercapturic acid pathway following the initial conjugation of isothiocyanates with GSH is enzyme-catalysed. Therefore, it can be assumed that this reaction is also catalysed by enzymes in insect herbivores. Thus, we can conclude that in the insect metabolism of isothiocyanates enzymes other than GSTs are involved, whose properties remain to be studied.

Both GSH- and CysGly-conjugates were formed from isothiocyanates of different structures in all five species investigated. The chosen species (*Spodoptera littoralis*, *S. exigua*, *Helicoverpa armigera*, *Mamestra brassicae*, *Trichoplusia ni*) cover a range of generalist lepidopteran caterpillars with increasing preference for brassicaceous plants.

The fact that they all appear to metabolise a range of isothiocyanates in essentially the same way suggests that conjugation of isothiocyanate with GSH is a general detoxification strategy in generalist caterpillars. In this respect, metabolism of glucosinolate hydrolysis products in generalists is as broad as glucosinolate metabolism in the specialists, *P. rapae* or *P. xylostella*, whose detoxification systems accept all glucosinolates tested as substrates (Ratzka *et al.*, 2002; Burow *et al.*, 2006a). Yet, in contrast to the specialist larvae, whose adaptations are powerful enough to cope with high doses of glucosinolates, the success of this generalist GSH conjugate-forming detoxification mechanism depends on the concentration of glucosinolates in the food plant, the catalytic activity of the myrosinases present, and the amounts of plant material ingested. Experiments using artificial diets spiked with isothiocyanates demonstrated that the capacity of *S. littoralis* detoxification is limited (see Chapter III). Excretion of free isothiocyanate in the faeces was linear with the amount ingested at low isothiocyanate concentrations yet reached a saturation level at high isothiocyanate concentrations. At these high concentrations, caterpillars also experienced severe toxic effects, probably as a result of the excess of isothiocyanate which was not detected in the faeces but retained in the organism. Other studies have investigated the effects of isothiocyanates on neonate lepidopteran larvae (Wadleigh & Yu, 1988; Li *et al.*, 2000; Agrawal & Kurashige, 2003) employing isothiocyanate concentrations in the same range as those used here. The toxic effects reported were at least as severe as those noted here, confirming the acute toxicity of isothiocyanates to lepidopterans. The effects described in these publications and in a study by Burow *et al.* (2006b) were also concentration-dependent. The results from this thesis provide an explanation for this concentration dependency by showing that at high concentrations of isothiocyanate in the diet, at which toxic effects severely impair caterpillar development and survival, free isothiocyanate is no longer excreted in amounts proportional to those ingested.

In Chapter I of this thesis it was shown that ingestion of plants producing nitriles instead of isothiocyanates from the same parent glucosinolates results in a decreased excretion of GSH conjugates. GSH conjugates with nitriles were not detected. In chemical syntheses, it was also not possible to conjugate GSH with nitriles, indicating that nitriles must be metabolised in some other way *in vivo*. In *P. rapae*, nitriles of aromatic glucosinolates produced by the actions of NSP are further metabolised to yield hippuric acids, sulphated nitriles or carboxylic acids (Agerbirk *et al.*, 2006; Vergara *et al.*, 2006; Vergara *et al.*, 2007; Agerbirk *et al.*, 2010). In *S. littoralis* and *S. exigua*, the hippuric acid conjugate of benzyl glucosinolate (i.e. of benzyl cyanide) was detected (I. Winde, pers. communication), suggesting that the metabolism of aromatic nitriles proceeds similarly in both generalist and specialist lepidopterans.

To elucidate how important GSH conjugation is in lepidopteran generalists, other means of tracing the progress of isothiocyanate metabolism need to be employed. Since the timing of glucosinolate hydrolysis is critical to how isothiocyanates and other hydrolysis products can be metabolized, experiments with artificial diets containing isothiocyanates can never fully simulate what happens when insects feed on intact plants. Not only should experiments be conducted with intact plants when possible, but studies with genetically modified plants are especially useful. Several studies have made use of the *Arabidopsis thaliana* transformants available, where aspects of the glucosinolate-myrosinase defence system have been manipulated. They showed for instance that *S. littoralis* caterpillars develop better when feeding on nitrile-producing rather than isothiocyanate-producing lines (Burow *et al.*, 2006b). *A. thaliana* mutants knocked out in myrosinases are better hosts than wild-type plants to several species of insect herbivores (Barth & Jander, 2006). Studies with mutants lacking aliphatic or indole glucosinolates or lacking both classes of glucosinolates showed that glucosinolates influence larvae of both specialist and generalist lepidopteran herbivores (Muller *et al.*, 2010). Generalist caterpillar development mostly benefitted from decreased glucosinolate contents, regardless which class of glucosinolates was reduced. Specialists, on the other hand, were mostly unaffected developmentally. But the absence of various glucosinolate types made a big difference for feeding behaviour and oviposition. *A. thaliana* mutants with altered glucosinolate hydrolysis, modified glucosinolate levels, different glucosinolate composition, or changes in myrosinase activity could reveal a lot about glucosinolate metabolism in generalist caterpillars if used in insect feeding studies with proper controls (wild type plants).

A different means of elucidating isothiocyanate and glucosinolate metabolism in herbivores is the use of radioactively or isotope-labelled glucosinolates and following their metabolic fate. In *P. rapae* this technique has helped unravel how these lepidopterans divert hydrolysis from isothiocyanates to nitriles (Wittstock *et al.*, 2004). For showing whether conjugation with GSH is a major route of isothiocyanate metabolism, labelled glucosinolates may be the key. This method could also be used to determine the metabolism of any other glucosinolate hydrolysis product. The disadvantages of this method include the major effort needed to prepare and purify labelled glucosinolates, and that of offering them to herbivores in a biologically-realistic manner with myrosinase.

Another option to investigate the contribution of GSTs and GSH to the conjugation process is a systematic analysis of the enzymatic properties of all individual GSTs present in caterpillar guts, by isolating the genes, heterologously overexpressing the encoded proteins and characterising them. If there are one or several GSTs especially suited to catalyse the conjugation of isothiocyanates to GSH, they might be identified in this way, as in Dabrowska et al. (2009).

Such information would be valuable in determining the function of GSTs in herbivore metabolism of glucosinolates. The existing evidence for mammalian cells is still equivocal. Several studies support the importance of GSTs in isothiocyanate metabolism. For example, cellular uptake of isothiocyanates is directly correlated with the amounts of available GSH in cells (both murine and human cell lines), and the more GSH available, the faster isothiocyanates can enter cells (Zhang & Talalay, 1998; Zhang, 2000; Zhang, 2001). Furthermore, in a direct comparison of human cells which contained low amounts of GSTs to ones which were transformed to overexpress GSTs, isothiocyanate uptake was enhanced in the presence of higher enzyme levels (Zhang, 2001). This points at an active role of GSTs in this process. However, this effect could only be shown for the initial phase of isothiocyanate uptake. When cells were incubated with isothiocyanates for more than 30 min up to 24 h, there was no difference in accumulated isothiocyanate between GST-containing and GST-free cells. The fact that the effect can only be observed in the first 30 min of isothiocyanate accumulation was correlated with the export of conjugates (Zhang, 2001). Finally, several studies have demonstrated that GSTs from a range of different organisms are capable of catalysing isothiocyanate conjugation to GSH (Kolm *et al.*, 1995; Meyer *et al.*, 1995; Nutricati *et al.*, 2006; Wiktelius & Stenberg, 2007). Hence, there is a basis for GST-catalysed conjugation of isothiocyanates.

The involvement of GSTs in isothiocyanate metabolism in insect herbivores is not supported by the results in Chapter II, which showed that GST-mediated catalysis was not detectable above the non-enzymatic background under conditions typically found in cells and in the midgut lumen. This implies that the spontaneous non-enzymatic reaction of GSH with isothiocyanates is responsible for conjugate formation instead of GSTs. In either case, GSH plays an important role in the process as illustrated by the results in Chapter III. When cellular GSH was depleted with BSO (*L*-buthionine-*R*,*S*-sulfoximine) prior to feeding on isothiocyanates, caterpillars excreted less GSH conjugates and developed more slowly than larvae whose GSH stores had not been depleted. In addition, caterpillars whose GSH-stores were enhanced by administration of *N*-acetylcysteine (a GSH-precursor) excreted higher amounts of GSH conjugates on isothiocyanate-containing diet. They also gained more weight compared to untreated and BSO-larvae. This implies that GSH is directly correlated to detoxification capacity. Recently, it has been shown that GSTs may have functions other than in xenobiotic metabolism (Frova, 2006; Dourado *et al.*, 2008; Allocati *et al.*, 2009; Dabrowska *et al.*, 2009; Morel *et al.*, 2009). Therefore, coupled with their role as phase II detoxification enzymes, GSTs probably play a variety of roles in *S. littoralis* in keeping with the diversity observed (Fig. 2, Chapter II). However, our results do not support a major role for GSTs in isothiocyanate metabolism.

The analysis of caterpillar faeces (see Chapter III) revealed that the amount of isothiocyanate-containing diet ingested is related to the excretion of isothiocyanates, except that at higher doses proportionally less was excreted. This suggests that isothiocyanate metabolism may be capacity-limited. Based on the results from Chapters II (Fig. 3) and III (Fig. 9), the metabolic capacity might be more dependent on GSH availability than GST activity. There is evidence in the literature showing that pharmacological processing of isothiocyanates in rats is capacity-limited (Ji *et al.*, 2005; Hanlon *et al.*, 2008): Ji *et al.* (2005) demonstrated that with increasing isothiocyanate concentration, half-life in the blood plasma of rats increased in a non-linear fashion. The authors suggest that this may be due to a number of reasons, including saturation of GSTs or the depletion of cellular GSH stores. The extended half-life may lead to increased free isothiocyanate levels circulating in the organism, and to a wider distribution of isothiocyanates in the body. While in this study noxious effects of high isothiocyanate doses were not reported, the ability of high isothiocyanate concentrations to cause reduced growth, slow development and even death when processing capacity was limited was vividly demonstrated in Chapter III.

Combining the results presented in this thesis and recently published investigations, a model for the metabolism of isothiocyanates in insect herbivores is proposed (Fig. 1). Based on our findings from GST enzyme assays (see Chapter II) and GSH measurements in gut lumen and epithelium (see Chapter III), we believe that the conjugation process is mainly confined to the epithelial cells of the midgut. As an insect ingests isothiocyanates, initially GSTs will participate in conjugation of isothiocyanates (Fig. 1A), as has been described for the first 30 min of isothiocyanate accumulation in human cells (Zhang, 2001). But because of the low catalytic efficiency of GSTs (Meyer, 1993; Meyer *et al.*, 1995) and the low velocity of enzymatic to non-enzymatic conjugation at intracellular conditions (Chapter II; (Wadleigh & Yu, 1988; Meyer *et al.*, 1995), spontaneous conjugation will be responsible for a much greater proportion of the resulting conjugates (Fig. 1B). The continued conjugation of isothiocyanates with GSH eventually depletes glutathione stores (Chapter III), because the conjugates are actively transported out of the cells (Zhang, 2000; Zhang *et al.*, 2005). For some time, *de novo* synthesis of GSH and the conversion of GSSG to GSH can slow down glutathione depletion (Griffith & Mulcahy, 1999). As GSH levels decrease, there may be an increase in enzymatic *vs*. non-enzymatic conjugation, as shown under low GSH concentration in Chapter II (Fig. 1C). But regardless of the mode of conjugate formation, as glutathione depletion becomes even greater, the GSTs are likely to retain their conjugate products to a greater degree (Meyer, 1993), preventing their dissociation and release of isothiocyanates.

At low GSH concentrations, enzymatic as well as non-enzymatic conjugation continues (Fig. 1D) because free isothiocyanates are continuously supplied both from the gut lumen and from isothiocyanates re-released from unstable conjugates. This leads to an extreme and long-lasting GSH depletion in the cell. As was shown in Chapter III, this can cause delayed development, growth inhibition and death.

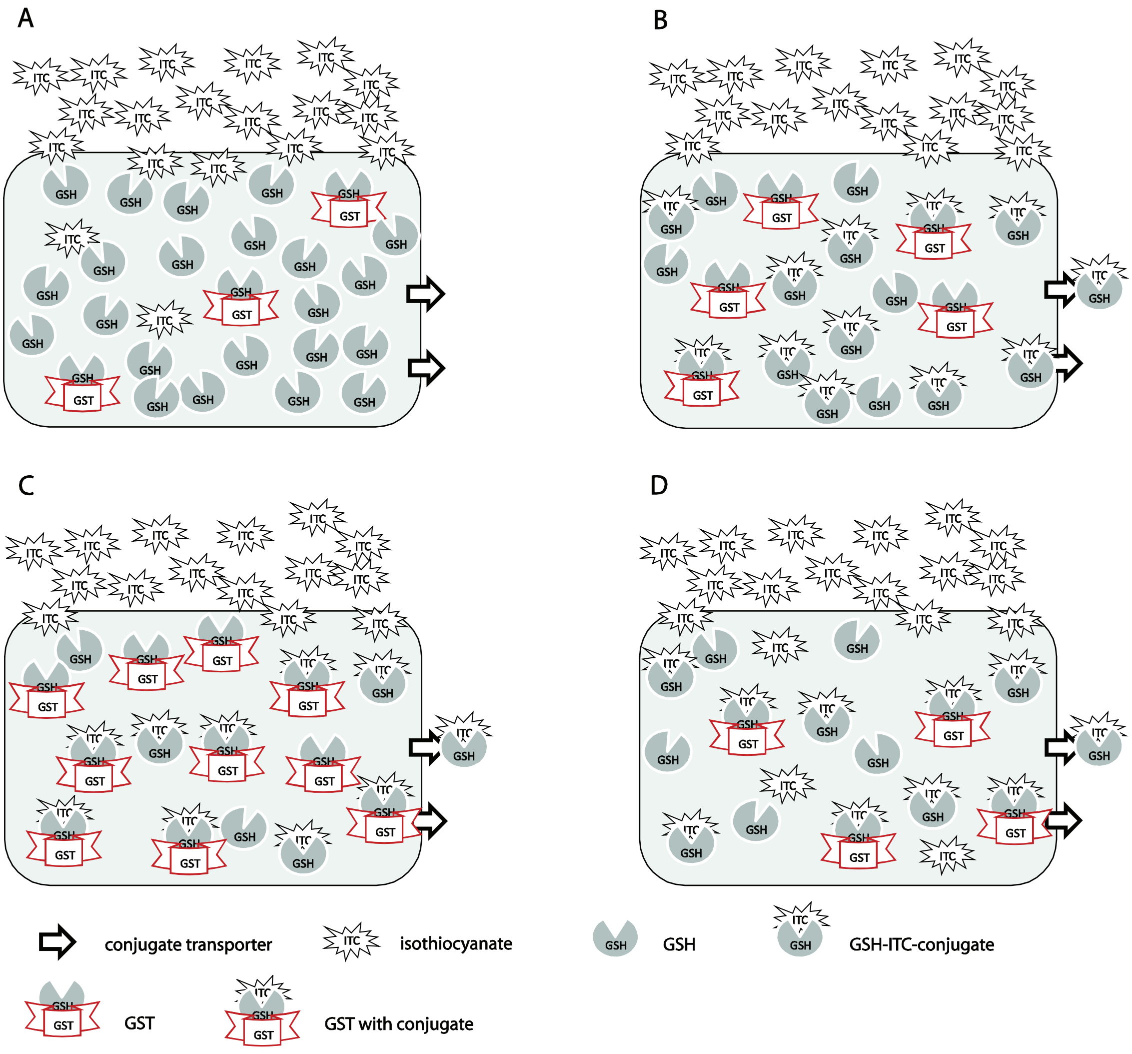


Fig. 10: Proposed model for isothiocyanate conjugation in generalist insect herbivores.

A) When an insect starts feeding on plant tissue containing glucosinolates, GSH concentrations are high. However, a steady influx of isothiocyanates leads to GST-catalyzed isothiocyanate conjugation.

B) Because of the high velocity of non-enzymatic conjugation at intracellular conditions, spontaneous conjugation will constitute the major route for formation of the resulting conjugates. Conversion of GSSG to GSH and the *de novo* synthesis of GSH provide new glutathione.

C) Eventually, GSH levels drop, leading to an increase in enzymatic conjugation since GST catalysis works more efficiently relative to non-enzymatic conjugation processes when GSH concentration is low. Strong GSH depletion ensues, followed by conjugate retention in GSTs and interaction of GSTs with conjugate transporters removing conjugate from the cell.

D) Low enzymatic as well as non-enzymatic conjugation continues, since under intracellular conditions the conjugates tend to dissociate, releasing free isothiocyanate. The constant supply of free isothiocyanate in the cell leads to an extreme and long-lasting GSH depletion. This massive depletion leads to damage by oxidative stress and direct harm from the free isothiocyanates.

In summary, the research in this thesis demonstrated that metabolism of isothiocyanates, the most commonly encountered glucosinolate hydrolysis products, proceeds by conjugation with GSH in generalist lepidopteran herbivores. No evidence was found for the formation of glucosinolate metabolites previously reported for specialist herbivores. Enzyme assays using partially purified GSTs from *Spodoptera littoralis* midguts showed that these enzymes can catalyse the conjugation of isothiocyanates to GSH *in vitro* under specific conditions. However, when assay conditions imitated the physiological environment expected in the midgut epithelial cells or the gut lumen, non-enzymatic conjugation of GSH to isothiocyanates occurred at a higher rate than GST-mediated conjugation, suggesting that this may be the dominant mechanism for isothiocyanate metabolism in these insects. Independent of how GSH-isothiocyanate conjugates are formed, limiting the supply of GSH was shown to diminish conjugate synthesis, as well as isothiocyanate excretion and insect performance, thus attesting the importance of conjugate production to survival on glucosinolate-containing plants. These results were combined in a new model for isothiocyanate metabolism in generalist lepidopteran herbivores which puts more emphasis on the availability of GSH rather than GST activity for successful isothiocyanate detoxification. Despite this new information on isothiocyanate metabolism, the mode by which isothiocyanates exert their toxicity on insect herbivores is still unknown. Increased knowledge of isothiocyanate mode of action in the future is necessary to appreciate the significance of isothiocyanate metabolism.

6 Summary

The glucosinolate-myrosinase system is an activated defence system in plants of the order Brassicales. Glucosinolates are the non-toxic precursors which are hydrolysed by myrosinases upon tissue damage yielding products such as isothiocyanates that act against a wide variety of attackers, including herbivores. Some lepidopterans specialised on brassicaceous plants possess biochemical adaptations, preventing the formation of isothiocyanates. Yet, no such mechanisms are known for generalist lepidopterans which also occasionally feed on plants of the Brassicales.

Here, analysis of the faeces of generalist *Spodoptera littoralis* larvae revealed that this species forms glutathione (GSH) and cysteineglycine conjugates of glucosinolate-derived isothiocyanates from the food plant. In the same way, the faeces of several other species of generalist caterpillars (*S. exigua*, *Helicoverpa armigera,* *Mamestra brassicae* and *Trichoplusia ni*) contain such conjugates of host plant-derived isothiocyanates. The caterpillars of all species investigated are able to form the conjugates from a large variety of isothiocyanate structures. This, and the fact that the tested species show an increasing preference for brassicaceous plants, suggests that conjugation with GSH is a generalist detoxification strategy in lepidopteran herbivores.

In mammals, isothiocyanates are known to be metabolised via the mercapturic acid pathway. In the first step, they are conjugated with GSH, a reaction which may be catalysed by mammalian glutathione-*S*-transferases (GSTs). The presence of GSH conjugates in larval faeces after consumption of crucifers suggests isothiocyanate-detoxification via GST activity in caterpillars. In this thesis, active native GSTs were purified from midguts of *S. littoralis* larvae. Under conditions widely used for GST characterisation, these *S. littoralis*-GSTs were capable of catalysing the conjugation reaction of GSH with different isothiocyanates. Yet in *in vitro* assays using physiological conditions simulating the environment of possible *in vivo* conjugation locations, GST activity was not detectable. We thus believe that GSTs do not play a major role in isothiocyanate metabolism in *S. littoralis* larvae.

Feeding tests with *S .littoralis* on diet containing defined amounts of 2-phenylethyl-isothiocyanate showed that isothiocyanate toxicity is structure- and dose-dependent. Analysis of larval faeces from these experiments revealed that isothiocyanate metabolism to GSH conjugates is capacity-limited: high concentrations of isothiocyanate lead to non-linear excretion of free isothiocyanate, accompanied by poor caterpillar performance. Determination of midgut GSH levels showed that consumption of isothiocyanates depletes intracellular GSH stores in *S. littoralis*. Caterpillars on isothiocyanate-containing diet whose GSH levels were pharmacologically enhanced or reduced compared to normal, excreted larger and smaller amounts

of metabolites respectively than control larvae. These findings suggest that isothiocyanate metabolism is directly correlated to GSH availability.

In conclusion, isothiocyanate conjugation with GSH appears to be a general metabolic strategy in generalist lepidopteran herbivores. A contribution of GST enzymes to isothiocyanate conjugation could not be demonstrated *in vitro* when physiological conditions in either the midgut cells or the midgut lumen were simulated. A central role for GSH in detoxification capacity and isothiocyanate metabolism is suggested.

7 Zusammenfassung

Das Glucosinolat-Myrosinase-System ist ein aktiviertes chemisches Abwehrsystem in Pflanzen der Ordnung Brassicales. Glucosinolate sind nicht-giftige Vorstufen, die in einer hydrolytischen Katalyse durch Myrosinasen aktiviert werden. Bei Gewebeverletzung werden oft giftige Isothiocyanate freigesetzt, die gegen eine Vielzahl von Angreifern, z.B. Herbivore, wirksam sind. Auf Brassicaceen spezialisierte Lepidopteren besitzen biochemische Anpassungen, die die Entstehung von Isothiocyanaten verhindern. Solcherlei Mechanismen sind bei generalistischen Lepidopteren, die nur gelegentlich an Kreuzblütlern fressen, jedoch unbekannt.

Spodoptera littoralis-Raupen sind polyphage Herbivoren. In dieser Arbeit zeigte die Analyse von *S. littoralis*-Kot, daß in den Raupen Konjugate gebildet werden die aus Isothiocyanaten und Glutathion oder Cysteinglycin bestehen. Die Isothiocyanate sind von den in Pflanzen als Sekundäre Pflanzeninhaltsstoffen vorhandenen Glycosinolaten abgeleitet. Auch der Kot verschiedener anderer Arten generalistischer Raupen (*S. exigua*, *Helicoverpa armigera*, *Mamestra brassicae* and *Trichoplusia ni*) enthält solche Konjugate mit Isothiocyanaten aus der Wirtspflanze. Die Raupen aller untersuchten Arten sind in der Lage, Konjugate mit einer Vielfalt strukturell unterschiedlicher Isothiocyanate zu bilden. Da diese Arten trotz ihrer polyphagen Lebensweise außerdem eine ansteigende Präferenz für Kreuzblütler zeigen, deuten diese Ergebnisse darauf hin, dass die Konjugierung von Isothiocyanaten mit GSH eine generelle Entgiftungsstrategie in Lepidopterenlarven ist.

In Säugetieren werden Isothiocyanate über den Mercaptursäureweg verstoffwechselt. Im ersten Schritt werden die Isothiocyanate mit GSH konjugiert, eine Reaktion, die von Glutathion-*S*-Transferasen (GST) katalysiert werden kann. Die Ausscheidung von GSH-Konjugaten mit dem Kot bei Raupen, die an Kreuzblütlern gefressen haben, weist darauf hin, dass Isothiocyanatentgiftung in diesen Insekten über GST-Aktivität erreicht werden könnte. In dieser Arbeit wurden aktive native GST aus dem Mitteldarm von *S. littoralis*-Raupen isoliert. Diese *S. littoralis*-GST können die Konjugierung von verschiedenen Isothiocyanaten mit GSH unter Bedingungen katalysieren, die weithin zur allgemeinen Charakterisierung von GST verwendet werden. Wenn *in vitro* jedoch die Bedingungen der möglichen *in vivo* Konjugierungsorte (Darmzellen, Darmlumen) simuliert wurden, konnte keine GST-Aktivität gemessen werden. Deswegen wird die Hypothese aufgestellt, dass GST für den Metabolismus von Isothiocyanaten in *S. littoralis* nicht entscheidend sind.

Faßversuche mit *S. littoralis* auf Diät, die definierte Mengen 2-Phenylethyl-Isothiocyanat enthielt, zeigte, dass die Giftigkeit von Isothiocyanaten struktur- und dosisabhängig ist. Eine Analyse des Raupenkots offenbarte, daß die Verstoffwechselung von Isothiocyanaten kapazitätsbeschränkt ist:

Aufnahme hoher Isothiocyanatkonzentrationen führte zu nicht-linearer Ausscheidung von freiem Isothiocyanat, gekoppelt mit einer schlechten Entwicklung der Raupen. Bei der Messung der GSH-Konzentration im Mitteldarmgewebe der Raupen zeigte sich, daß die Aufnahme von Isothiocyanaten zur Leerung der intrazellulären GSH-Speicher führt. Wenn Raupen, deren GSH-Levels pharmakologisch erhöht oder reduziert worden waren, auf Isothiocyanat-haltiger Diät fraßen, schieden sie im Vergleich zu nicht-manipulierten Kontrolltieren entsprechend höhere oder niedrigere Mengen an Metaboliten aus. Diese Beobachtungen lassen darauf schließen, daß Isothiocyanatentgiftung direkt mit der Verfügbarkeit von GSH zusammenhängt.

Zusammenfassend scheint die Konjugierung mit GSH in generalistischen Raupen eine allgemeine Strategie der Verstoffwechselung von Isothiocyanaten zu sein. Ein Beitrag von GST-Enzymen zu dieser Reaktion konnte nicht gezeigt werden, wenn die *in vitro*-Bedingungen die Situation in Darmzellen oder im Darmlumen simulierten. Daher wird stattdessen für GSH eine zentrale Rolle im Metabolismus von Isothiocyanaten sowie für die Entgiftungskapazität der Raupen angenommen.

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10 Curriculum Vitae

PERSONAL

Name	Katharina Schramm
Address	St.-Jakob-Str. 12 07743 Jena
e-mail	kschramm@ice.mpg.de

EDUCATION

12/2004-today	PhD position at the Max Planck Institute for Chemical Ecology, Department of Biochemistry, Jena, Germany
01/2004	Diploma at the Free University Berlin Thesis completed at the lab of Prof. Dr. M. Wink, Institut für Pharmazie und Molekulare Biotechnologie, University Heidelberg Title: Insecticidal properties of sparteine and its natural and artificial derivatives to *Oncopeltus fasciatus*, *Drosophila melanogaster* and *Sitophilus granarius*
10/97-01/2004	Studying biology at the universities of Bonn, Berlin (Free University) and Helsinki
07/97	Abitur Beethoven Gymnasium Bonn, graduation date: 13.06.1997

PRACTICAL AND LABORATORY EXPERIENCE

09/2004-today	Member of the International Max Planck Research School (IMPRS) on “The Exploration of Ecological Interactions with Molecular and Chemical Techniques”
03/2002-03/2003	Research assistant (HiWi) at the Institut für Ökologie of the TU Berlin in the lab of Prof. Dr. J. Erber: Habituation and learning sensitivity of *Drosophila melanogaster* and *Apis mellifera*
05/2001	Volunteer at the Institut für Angewandte Zoologie/ Ökologie der Tiere of the Free University Berlin in the lab of Dr. J. Ruther: Identification and analysis of the sex pheromone of the cockchafer *Melolontha hippocastani*
09/99-12/99	Research assistant of Dr. J. Protomastro at Iguazú National Park, Puerto Iguazú, Argentina, funded by a travel grant of the DAAD

11 Publications & Presentations

Harvey, JA, Biere, A, Fortuna, T, Vet, LEM, Engelkes, T, Morrien, E, Gols, R, Verhoeven, K, Vogel, H, Macel, M, Heidel-Fischer, HM, Schramm, K & van der Putten, WH (2010). Ecological fits, mis-fits and lotteries involving insect herbivores on the invasive plant, *Bunias orientalis*. Biological Invasions **12**(9): 3045-3059.

Oral presentations:

- 6th Kurt Mothes Workshop Secondary Metabolites, Sept. 2008, Jena, Germany
- 25. Meeting of the International Society for Chemical Ecology (ISCE), Aug. 2008, Penn State University, State College, USA (funded by an IMPRS Travel Award)
- International Congress of Entomology (ICE), July 2008, Durban, South Africa
- 7th Biannual IMPRS Symposium, Jena, Germany (Award for Best Oral Presentation)

Poster presentations:

- Second International Conference on Glucosinolates, Mai 2009, Elsinore, Denmark
- 13th Symposium in Insect-Plant Interactions (SIP 13), Aug. 2007, Uppsala, Sweden (funded by a DFG Travel Grant, SCHR 1199/1-1)
- 23rd Annual ISCE Meeting, July 2007, Jena, Germany
- First International Conference on Glucosinolates, Sept. 2006, Jena, Deutschland
- IMPRS Symposia 2006, 2007, 2009

12 Selbständigkeitserklärung

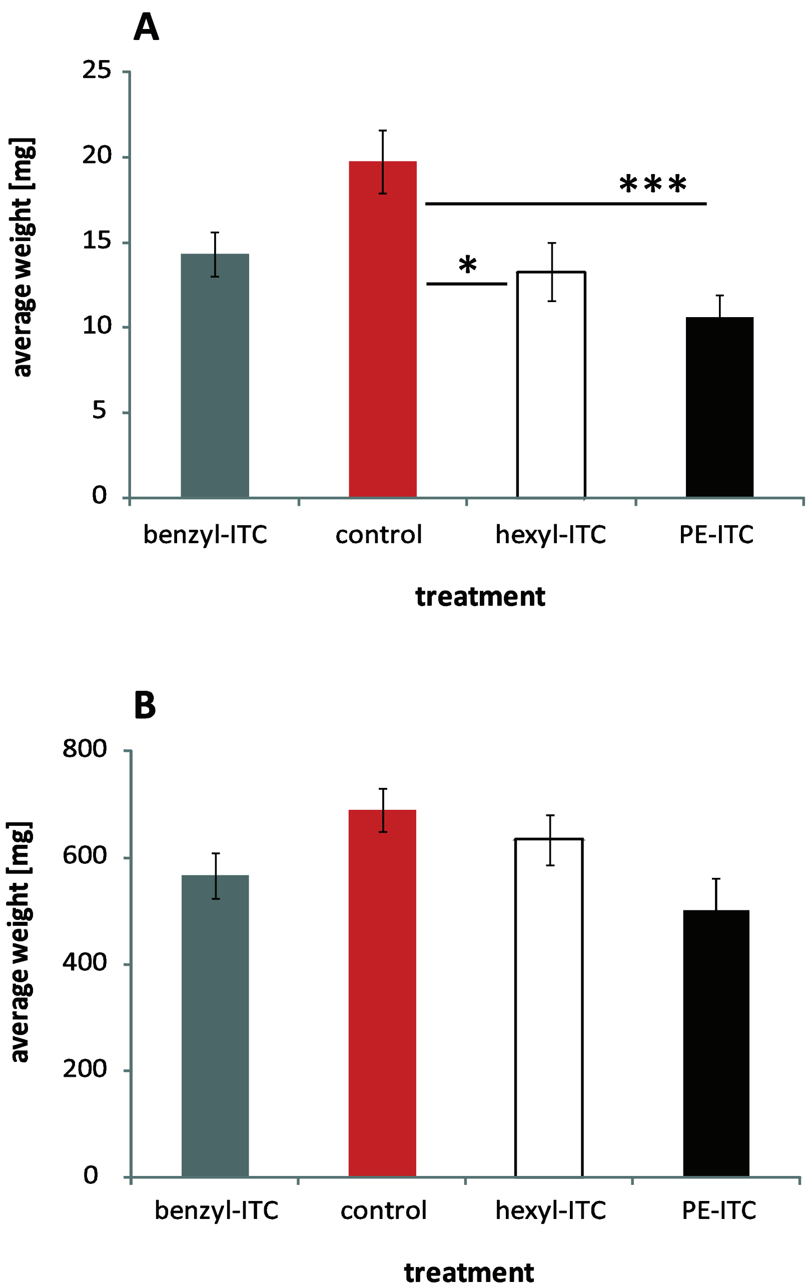
Die zurzeit gültige Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität ist mir bekannt. Die vorliegende Arbeit wurde von mir selbst und nur unter Verwendung der angegebenen Hilfsmittel erstellt und alle benutzten Quellen angegeben. Alle Personen, die an der experimentellen Durchführung, Auswertung des Datenmaterials oder bei der Verfassung der Manuskripte beteiligt waren, sind benannt. Es wurde weder bezahlte noch unbezahlte Hilfe eines Promotionsberaters in Anspruch genommen.

Die vorliegende Arbeit wurde bisher weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch bei einer anderen Hochschule als Dissertation eingereicht.

Katharina Schramm

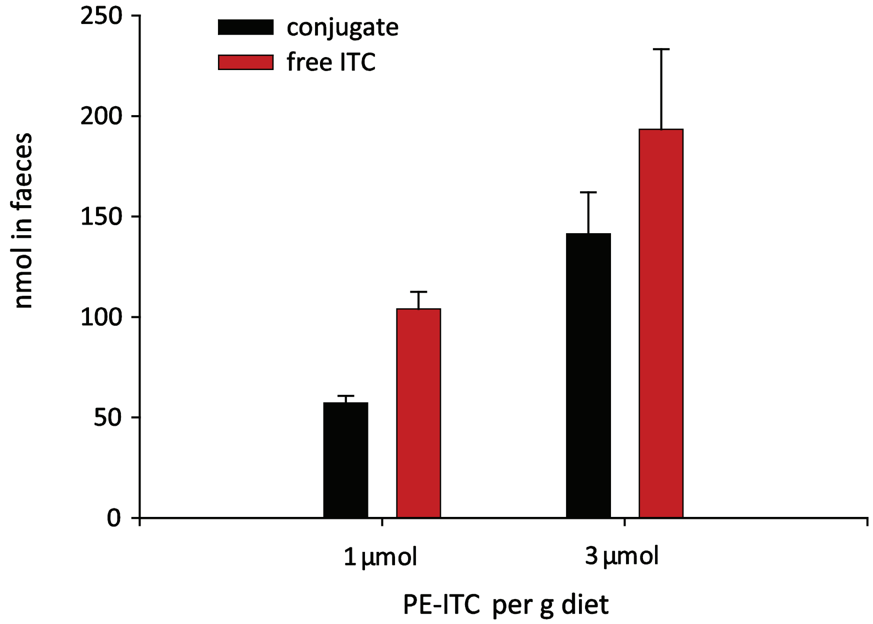
Jena, November 2010

Scientific Appendix



Supplemental Fig. S1: Average weights of *S. littoralis* caterpillars on selected days on 2 µmol isothiocyanate per g diet.

Newly moulted second instar larvae were placed on artificial diet containing no isothiocyanate (control) or 2 µmol of benzyl-, hexyl- or PE-isothiocyanate per g diet. Individual weight was recorded daily. Differences in mean weight were significant for hexyl- and PE-ITC on day 12 of the development (A). Mean weights did not differ significantly on day 21 of the development (B).



Supplemental Fig. S2: Non-linear excretion of free isothiocyanate in *S. littoralis* larvae on 3 µmol PE-ITC per g diet.

Caterpillars feeding on 3 µmol*g^{-1} PE-ITC excreted three times more GSH metabolites than caterpillars feeding on 1 µmol*g^{-1}. Yet the excretion of free isothiocyanate in the faeces is non-linear: larvae feeding on 3 µmol*g^{-1} PE-ITC excreted roughly twice as much free isothiocyanate than caterpillars feeding on 1 µmol*g^{-1}.